Stromal cell function in non-pregnant endometrium and decidua of early pregnancy

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Declaration

Except where due acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. The work described in this thesis has not been previously accepted for, or is currently being submitted in candidature for another degree.

In Chapter 3, I acknowledge the assistance of Prof Jan Brosens and Marius Jones, in whose lab I performed the steroid response element reporter/promoter assay. Additionally, Marius Jones performed the siRNA knockdown of SMAD 4, the data from which was analysed in Figures 3.25, 3.26 and 3.27. Marius Jones also performed the Western blot which analysed the SMAD 4 silencing efficiency (Figure 3.26), the Western blot which analysed the protein expression of PR and PIASγ in non-decidualised and decidualised cells (Figure 3.14) and the Western blot analysing the protein expression of p-SMAD 2 in non-decidualised and decidualised cells (Figure 3.22).

In Chapter 4, I acknowledge the assistance of Prof Jan Brosens and Marius Jones, in whose lab I performed the reporter/promoter assay to analyse the effect of TGFβ1 on the transactivation potential of the decidual prolactin promoter. Additionally, Marius Jones performed the siRNA knockdown of SMAD 4, the data from which was analysed in Figure 4.20.

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Abstract

The human endometrium is a highly specialised tissue, which provides an optimal environment for the establishment and maintenance of a successful pregnancy. The endometrium undergoes remarkable cycles of remodelling, involving proliferation, differentiation, including decidualisation of the stroma, and in the absence of pregnancy, breakdown and repair. The menstrual cycle is governed by the steroid hormones oestrogen and progesterone acting via their nuclear receptors, but at a paracrine level by a myriad of growth factors, cytokines and proteases. Decidualisation of the endometrium is essential to create an environment conducive to the implantation of a conceptus, and in the absence of fertilisation, is likely mandatory for the onset of menstruation. Decidualisation is also associated with a unique immune environment, characterised by the presence of large numbers of uterine-specific natural killer cells (uNK). uNK cells increase in number in secretory phase endometrial stroma, implying the control of progesterone on their expansion. However, they lack the nuclear progesterone receptor and growth and differentiation may depend on interactions with endometrial stromal cells (ESCs) and other hormones such as luteinising hormone (LH) and human chorionic gonadotrophin (hCG). Transforming growth factor β 1 (TGFβ1) is present in both epithelial and stromal cells within the endometrium at stages of the menstrual cycle consistent with rapidly proliferating or remodelling tissue and inflammatory events, and has been demonstrated to transduce progesterone action. Whether TGFβ1 influences decidualisation is unclear.

The aims of this research project have been to investigate the role of TGFβ1 in mediating factors involved in decidualisation, with particular regard to expression of nuclear progesterone receptor (PR), the Wnt antagonist, Dickkopf-1 (DKK), and markers of decidualisation in order to identify a potential role for TGFβ1 in decidualisation and menstruation, and to characterise further the uNK cell to determine how uNK cells may interact with other uterine hormones and ESCs during the secretory phase and early pregnancy.

In primary human ESC cultures, both non-decidualised and decidualised *in vitro,* PR mRNA and protein expression was initially augmented over 2 h then downregulated in a TGFβ1–specific and time-dependent manner. Furthermore, DKK expression levels were downregulated after TGFβ1 treatment in a biphasic manner in non-decidualised cells and over a period of 24 h in decidualised cells. However, the downregualtion of both PR and DKK, reported to be a progesterone-responsive gene, could not be attributed to TGFβ1 preventing gene transcription as TGFβ1 was without effect on progesterone or oestrogen binding to their respective response elements. In addition, TGFβ1 treatment of *in vitro* decidualised ESCs and stromal cells from 1st trimester decidua inhibited production of classical decidualisation markers, IGFBP-1, tissue factor and prolactin at both mRNA and protein levels, and suppressed the transactivation potential of the prolactin promoter by acting at the critical decidualisation region. SMAD4 knockdown employing siRNA techniques was inconclusive. Therefore, no data was obtained to elucidate whether TGFβ1 was acting via the SMAD signalling pathway or via alternative pathways e.g. Wnt, MAP kinase and JNK signalling.

Primary cultures of human uNK cells were used for studying the possibility that LH and hCG may be the hormones responsible for the influx and proliferation of uNK cells. LH/hCG receptors were not expressed on the uNK cells, however, mannose receptors, which have been proposed to bind hCG, were localised to uNK cells. Furthermore, when uNK cells were treated with hCG, colocalisation of hCG and mannose receptors was observed. Additionally, treatment with low doses of hCG inhibited IFNγ protein release from uNK cells derived from 1st trimester decidua, providing a functional role for hCG acting via the mannose receptor.

Further investigation into the interactions between the mannose receptor and LH/hCG would be important in clarifying the mechanisms controlling uNK influx and proliferation. The interactions between ESCs and uNK cells need to be clarified further to assess the roles of uNK cells in reproductive processes. This work has thus revealed a potential novel mechanism for the influx and recruitment of uNK cells mediated by the mannose receptor.

Presentations relating to this thesis

Transforming Growth Factor-Beta 1 regulates messenger RNA expression of nuclear progesterone receptor in Endometrial Stromal Cells

Poster presented at the 13th Simpson Symposium and the 7th International Conference on the ECM of the Female Reproductive Tract, Aug 2004

Transforming Growth Factor- Beta 1 regulates Nuclear Progesterone Receptor expression in Endometrial Stromal Cells: A novel model for studying menstruation *in vitro*?

Oral communication at the Munro Kerr conference, Feb 2005

Transforming Growth Factor Beta 1, an anti-progestin involved in menstruation?

Oral communication presented at the 14th Simpson Symposium, Aug 2005

TGFβ1 and Decidualisation

Poster presented at the 8th European Congress of Endocrinology, April 2006

TGFβ1 inhibits decidualisation of human endometrium and attenuates the activation of the decidual prolactin promoter via a SMAD4-independent pathway

Oral communication to be presented at the 8th international conference on the ECM of the female reproductive tract, Nov 2006

TGFβ1 attenuates expression of both the progesterone receptor and dickkopf in differentiated human endometrial stromal cells

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Papers in preparation

TGFβ1 attenuates expression of both the progesterone receptor and dickkopf in differentiated human endometrial stromal cells.

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TGFβ1 inhibits decidualisation of human endometrium and attenuates the activation of the decidual prolactin promoter.

Nicole Kane, Marius Jones, Jan Brosens, Rodney Kelly, Hilary Critchley

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Abbreviations

-/-	knock out mice
αSMA	α smooth muscle actin
βA/βB	Activin subunits A/B
AA	arachidonic acid
ABC	avidin biotin peroxidase detection system
AF-1/2	activation functional domain 1/2
ANOVA	analysis of variance
APC	adenomatous polyposis coli
AR	androgen receptor
ARE	androgen response element
ART	assisted reproductive technology
AS	adenylyl cyclase
ATP	adenosine triphosphate
α-2 M	α-2 macroglobulin
bFGF	basic fibroblast growth factor
BMP	bone morphogenic protein
BSA	bovine serum albumin
BUS	B upstream sequence
C	catalytic units
Ca^{2+}	calcium
$CaCl_2$	calcium chloride
cAMP	adenosine-3',5'-cyclic monophosphate
CBP	CREB binding protein
CD40L	CD40 ligand
cDNA	complimentary DNA
CEBP	CCAAT/enhancer binding protein
CL	corpus luteum
Co-SMAD	common SMAD
COX-1/2	cyclooxygenase-1/2
CR	Cysteine rich domain

CRE	cAMP response element
CREB	CRE binding protein
CREM	CRE modulator
CSF-1	colony stimulating factor-1
CSM	cell separation medium
Ct	threshold cycle
CTLDs	C-type lectin-like carbohydrate recognition domains
DAB	3,3'-diaminobenzidine
DBD	DNA binding domain
DCC	dextran-coated charcoal
DEPC	Diethyl pyrocarbonate
DEX	dexamethasone
DHT	dihydrotestosterone
DKK	dickkopf
DM	decidualising medium
DNA	deoxyribonucleic acid
DPP	decapentaplegic
dPRL	decidual prolactin
Dsh	dishevelled
DSC	decidual stromal cell from 1st trimester of pregnancy
E_2	Oestradiol
ECM	extracellular matrix
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme linked immuno absorbant assay
EP_{1-4}	PGE_2 receptors 1-4
ER-α/β	oestrogen receptor-α/β
ERE	oestrogen response element
ESC	endometrial stromal cell
ET(-1/2/3)	endothelin (-1/2/3)
$ET_{A/B}$	ET receptor $_{A/B}$
Eu	europium
FACS	fluorescent activated cell sorter

FAM	6-carboxy-fluorescein
FCS	foetal calf serum
FITC	fluorescein isothiocyanate conjugate
FNII	fibronectin type II repeats
FSH	follicle stimulating hormone
Fz	frizzled
g	relative centrifugal force
G α/β/γ	G protein α/β/γ
G-CSF	granulocyte-colony stimulating factor
GD	gestation day
Gi/Gs	G protein inhibitory/stimulatory
GM-CSF	granulocyte/macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GS	glycerine serine domain
GSK-3β	glycogen synthase kinase-3β
hCG	human chorionic gonadotrophin
HLA-DR	human leukocyte antigen
HRP	horse radish peroxidase
ICAM	inflammatory cell adhesion molecule
ICC/IHC	immuno cyto/histo chemistry
ICF/IHF	immuno cyto/histo fluorescence
IFN γ	interferon-γ
IGF	insulin-like growth factor
IGFBP-1/2 etc	insulin-like growth factor binding protein-1/2 etc
IgG	immunoglobulin
IL-1/2 etc	interleukin-1/2 etc
IL-1R type-1/2	IL-1 type 1/2 receptor
IL-T	immunoglobulin-like transcript
IP-10	interferon-inducible protein 10
IRF-1	interferon regulatory factor - 1

IUGR	intrauterine growth restriction
IVF	*in vitro* fertilisation
KIR	killer cell immunoglobulin-like receptors
KDR	Type 2 receptor of VEGF/ kinase domain receptor
LAP	latency-associated peptide
LBD	ligand binding domain
LDL	low density lipoprotein
LEF-1	lymphocyte enhancer factor
LGL	large granular leukocytes
LGR 7/8	relaxin receptors
LH	luteinising hormone
LIF	leukaemia inhibitory factor
LMP	last menstrual period
LNG-IUS	levonorgestrel-releasing intra uterine system
LRP 5/6	LDL-related proteins 5/6
LTGFβ	Latent TGFβ
luc	luciferase
MCP-1	monocyte chemoattractant protein -1
M-CSF	macrophage-colony stimulating factor
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
MIP-1α/β	macrophage inflammatory protein-1α/β
MIS	müllerian inhibiting substance (anti-müllerian hormone
Miso	Misoprostol
MPA	medroxyprogesterone acetate
MMP	matrix metalloproteinase
mRNA	messenger RNA
MRR	macrophage mannose receptor
NaCl	sodium chloride
NaN_3	sodium azide
NaOH	sodium hydroxide
NBF	neutral buffered formalin

NCAM	neural cell adhesion molecule
nGRE	non-consensus GRE
NGS	normal goat serum
NK	natural killer (cell)
NSB	non-specific binding
P	progesterone
PAI-1	plasminogen activator inhibitor-1
PB	peripheral blood
PBMC	peripheral blood monocyte cells
PBNK	peripheral blood natural killer cells
PBS(T)	phosphate buffered saline (tween)
p/CAF	p300/CBP associated factor
PCR	polymerase chain reaction
PDE	phophodiesterase
PE	phycoerythrin
PG	prostaglandin
PGD_2	prostaglandin D_2
PGDH	prostaglandin dehydrogenase
PGH_2	prostaglandin H_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostaglandin I_2
PIAS $\alpha/\beta/\gamma$	protein Inhibitor of Activated STAT $\alpha/\beta/\gamma$
PKA	protein kinase A
PLA_2	phospholipase A_2
PR A/B/C	progesterone receptor-A/B/C
PRE	progesterone response element
PRL	prolactin
PVDF	polyvinylidine difluoride
Q-RT-PCR	quantitative reverse transcription-polymerase chain reaction
R	regulatory units
RIA	radioimmunoassay

RLB	reporter lysis buffer
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RSB	RNA storage buffer
R-SMAD	receptor-regulated SMAD
RT	reverse transcription
RU486	mifepristone
SARA	SMAD anchor for receptor activation
SDF-1	stromal cell-derived factor -1
Sgy	soggy
sMMR	soluble MMR
siRNA	short hairpin RNA
SLPI	secretory leukocyte protease inhibitor
(p)SMAD 2 etc	(phosphorylated) mothers against decapentaplegic (MAD) and the *C. elegans* protein SMA 2 etc
SMPT	silencing mediator of retinoid and thyroid hormone receptor
SP (-1/3)	specificity protein (-1/3)
SPC	streptavidin peroxidase conjugate
SRC (-1)	steroid receptor coactivator (-1)
SSP	short signal peptide
STAT	signal transducers and activators of transcription
STOP	surgical termination of pregnancy
SUMO	small ubiquitin-related modifier
TAMRA	carboxy tetra methyl rhodamine
TB/NS	tris buffered/ NaCl saline
TCF	T cell factor
TF	tissue factor
LTGFβ	latent TGFβ
TGFβ	transforming growth factor-β
Th-1/2	T helper-1/2
TIMP	tissue inhibitor of MMP

TNF-α	tumour necrosis factor-α
TRIR	total RNA isolation reagent
TSH	thyroid stimulating hormone
TXA_2	thromboxane A_2
UtMVECs	uterine microvascular endothelial cells
uNK	uterine natural killer (cell)
u/tPA	urokinase/tissue type plasminogen activator
uPAR	uPA receptor
VEGF	vascular endothelial growth factor
Wnt	Wingless/INT-1 related

Chapter One

1 Literature Review

1.1 The Human Uterine Endometrium and the Menstrual Cycle

The human uterine endometrium is a unique, dynamic organ in its ability to undergo extensive and rapid remodelling, with cycling proliferation, differentiation, and degeneration. During every menstrual cycle, the endometrium regenerates and proliferates after menses; progressively and sequentially differentiates to prepare for successful implantation of a conceptus; and, if pregnancy fails to be established, breaks down and is shed during menstruation (Wynn and Jollie 1989). The endometrium is composed of two compartments, the basalis, which persists from cycle to cycle, and the transient superficial functionalis. Morphological changes during the menstrual cycle occur only in the functionalis and it is this layer which is shed during menstruation. Regeneration of the endometrium, after menstrual shedding, occurs from the underlying basalis. These well-documented changes occur over a 24 to 35 day period and are primarily governed by sequential exposure to the ovarian hormones, oestrogen and progesterone (Markee 1940; Noyes, Hertig et al. 1950; Buckley and Fox 1989).

The classic 28 day menstrual cycle is generally described in three phases, the proliferative phase (days 5-14), the secretory phase (14-28) and in the absence of implantation, menstruation (days 1-4) (Figure 1.1). The following descriptions of the changes occurring in the endometrium have been adapted from studies by Markee *et al*, Noyes *et al,* and Buckley *et al* (Markee 1940; Noyes, Hertig et al. 1950; Johannisson, Landgren et al. 1982; Johannisson, Parker et al. 1982; Buckley and Fox 1989).

1.1.1 The proliferative phase

The following description is based on the work of Markee *et al*, Li *et al*, Johannisson *et a*l and Wynn *et al* (Markee 1940; Wynn 1974; Johannisson 1985; Johannisson, Landgren et al. 1987; Li, Rogers et al. 1988; Wynn and Jollie 1989).

The proliferative phase follows menstruation and is characterised by re-epithelialisation and proliferation of endometrial glandular, stromal, and vascular endothelial cells, producing a notable thickening of the endometrium from about 1 mm to 3-4 mm by the time of ovulation (Glasser 2002). Oestrogen, regulated by follicle stimulating hormone (FSH), is the steroid hormone responsible for the changes observed during the proliferative phase. Under the influence of FSH, released from the adenohypophysis, a cohort of antral follicles begins to undergo preovulatory maturation. One of the follicles will become dominant, the Graafian follicle, and will eventually ovulate, secreting oestrogen. The increase in oestrogen levels exerts an inhibitory effect on the adenohypophysis, resulting in a decrease in FSH secretion below the minimum level that will support the growth of the other follicles; hence, the Graffian follicle further develops, with oestrogen levels continuing to rise. Once a threshold level is reached positive feedback on the adenohypophysis occurs resulting in the LH surge which is responsible for ovulation, usually observed approximately 16 h later (LH+0).

The proliferative phase is further categorised into early, mid, and late proliferative phase. The early proliferative phase (days 5-7) is usually identified by the formation of new glands which are typically straight, tubular in structure and somewhat undifferentiated, and the stroma, which resemble undifferentiated fibroblasts (More, Armstrong et al. 1974) contain spindly cells with large nuclei. The glands are lined by columnar epithelial cells with basally located nuclei. Occasionally mitotic figures are observed (Wynn and Jollie 1989). The mid proliferative phase (days 8-10) sees an increase in length and tortuosity of the glands with pseudostratified cells and prominent mitotic figures (Johannisson, Landgren et al. 1987). The stroma becomes highly vascularised. During the late proliferative phase (days 11-14) the glands

contain tall columnar cells and exhibit marked tortuosity and wider lumen (Johannisson, Landgren et al. 1987). Increased mitotic activity and the formation of pronounced nuclei are observed in the stroma in the 1 to 2 days preceding ovulation, LH-1 and LH-2 (Wynn and Jollie 1989).

1.1.2 The Secretory Phase

The following description is based on the work of Markee *et al*, Li *et al*, Johannisson *et a*l and Wynn *et al* (Markee 1940; Wynn 1974; Johannisson 1985; Li, Rogers et al. 1988; Wynn and Jollie 1989).

Following ovulation, the secretory phase is associated with morphological and functional changes evident in the luminal epithelium, glandular and stromal compartments and the vasculature (Dockery and Rogers 1989). These changes are primarily governed by circulating levels of progesterone, although oestrogen continues to be produced by the corpus luteum (Dockery and Rogers 1989). Under the control of LH the theca and granulosa cells of the follicle luteinise, forming the corpus luteum. The corpus luteum is responsible for the high level of progesterone in the secretory phase of the cycle. In the early secretory phase (Day 14-20), the level of progesterone rises rapidly and structural changes are observed in accordance (Dockery and Rogers 1989). The first sign that ovulation has occurred is the appearance of subnuclear vacuoles of glycogen in the glandular epithelium (day 16). These vacuoles displace the nuclei to the centre of the cells resulting in the alignment of nuclei. The glands become convoluted, accompanied by increased tortuousity and dilation. The stromal mitotic rate is still at a maximum. In the mid secretory phase (day 19 to day 23), the secretory activity of the glands becomes apparent with copious intraluminal secretions seen at around day 20. At this time the stromal changes represent the earliest phase of the cascade of differentative events leading to decidualisation. Focal stromal oedema peaks around day 22/23. In the late secretory phase (day 24 to day 28), there is differentiation of spiral arterioles and areas of oedematous stroma become more extensive. Perivascular stromal cells become more apparent as their cytoplasm increases in volume and their nuclei enlarge. These are predecidual cells and on day 25 the progressive spread of predecidual changes begins

under the surface epithelium forming a solid sheet of tissue. The stromal cells undergo a decidual reaction in preparation for implantation and progressively form the decidua during pregnancy.

Pinopodes are expressed on days 19-21 on the apical surface of the luminal uterine epithelium (Psychoyos 1992; Nikas, Drakakis et al. 1995). The timing of their appearance is progesterone-dependant and they appear around the time of implantation (Martel, Monier et al. 1991; Nikas, Drakakis et al. 1995; Nikas and Psychoyos 1997). Infiltration of leukocytes begins to occur around day 26. Although macrophages and lymphocytes accumulate, the major leukocyte cell type in the secretory phase is the uterine Natural Killer cell (King, Wellings et al. 1989; Trundley and Moffett 2004). These cells remain abundant in the first trimester of pregnancy and will be discussed in more detail in section 1.4.2. If pregnancy does not occur, glandular secretion becomes exhausted and the glands have a saw-toothed appearance. Growth of the endometrium ceases and dehydration results in shrinkage. Focal necrosis becomes apparent and menstruation begins (day 1).

1.1.3 Menstruation

In the absence of nidation, the superficial functional layer of the endometrium is discarded by the process of menstruation in response to declining progesterone levels because of corpus luteum demise (Henzl, Smith et al. 1972; Cornillie, Lauweryns et al. 1985). The withdrawal of progesterone converts the refractory uterus into a spontaneously steroid-responsive organ (Csapo and Resch 1979). Menstruation is the process of remodelling which prepares the endometrium for implantation of a blastocyst in the ensuing menstrual cycle. Endometrial breakdown and shedding is initiated at focal points, with areas of breakdown evident coincident with regeneration of neighbouring areas (Fraser 1990; Salamonsen and Woolley 1999). Menstruation occurs only in humans, Old World monkeys, anthropoid apes and two non-primate species, the elephant shrew (*Elephantus myuras jamesoni*) and some bats (e.g. *Glossophaga sorcinina*). Due to the terminal differentiation of the stroma during the menstrual cycle, in response to progesterone and independently of a blastocyst, menstruation must occur in order to allow regeneration of the

endometrium for the next cycle (Ferenczy 1976; Wynn and Jollie 1989). This may provide a maternal protective mechanism that counteracts the aggressive implantation occurring in menstruating species (Finn 1987; Finn 1996). Indeed, human trophoblast implantation is unique, exhibiting the greatest degree of trophoblast invasion seen in all species even amongst those with a hemochorial placenta, including the higher apes (Ramsey, Houston et al. 1976; Pijnenborg, Robertson et al. 1981; Bell 1990). Menstruation does not occur in mammals with less aggressive implantation (Bell 1990). For instance, in rodents, trophoblast cells adhere to the luminal epithelium but do not invade the endometrium. Trophoblast cells phagocytose the epithelial cells placing the blastocyst within the stroma compartment, after which the blastocyst becomes invasive and stromal changes occur (Pijnenborg, Robertson et al. 1981; Bell 1990). It is therefore unnecessary for these mammals to menstruate as the stromal reaction occurs only in the presence of pregnancy. This concept that menstruation is dependent on invasive implantation is further supported from the hormone-primed mouse model, in which no bleeding, nor decidualisation was observed with progesterone withdrawal (Finn and Pope 1984). However, when the hormone primed mouse uterus was stimulated with a drop of oil, mimicking the trophoblast and initiating decidualisation of the endometrium, and the progesterone source withdrawn, a menstruation-like response occurred (Finn and Pope 1984).

Knowledge of changes arising in the endometrium, both preceding and during menstruation is largely based on the elegant study by Markee undertaken almost 70 years ago (Markee 1940). Markee described a period of regression occurring 2-6 days before the onset of bleeding with the determination that progesterone and oestrogen are major controlling factors over these structural changes (Markee 1940). It was known that progesterone acts following oestrogen priming and then is withdrawn in the lead up to menstruation. The effects of these hormonal changes were studied in endometrial fragments transplanted into the eye of the rhesus monkey, where changes in the vasculature could be observed (Markee 1940). Striking changes were noted in the endometrial blood vessels. The arteries that supply the endometrium are straight when in the basalis region but become coiled within the functionalis (Markee 1940). As the secretory phase of the menstrual cycle progresses, the spiral arteries become

increasingly coiled until circulation slows and stasis occurs due to the increased resistance encountered in the vessels (Markee 1940). Approximately 4 to 24 hours prior to menstruation, vasoconstriction of the spiral arteries commences. This induces anoxia in the functionalis and results in bleeding. Leukocyte infiltration accompanies menstruation (Bulmer, Lunny et al. 1988; King, Burrows et al. 1998). Changes observed in the endometrium across the menstrual cycle are reviewed in Campbell *et al*, Salamonsen *et al* and Critchley *et al* (Campbell and Cameron 1998; Salamonsen, Kovacs et al. 1999; Critchley, Kelly et al. 2001) and summarised in Figure 1.1.

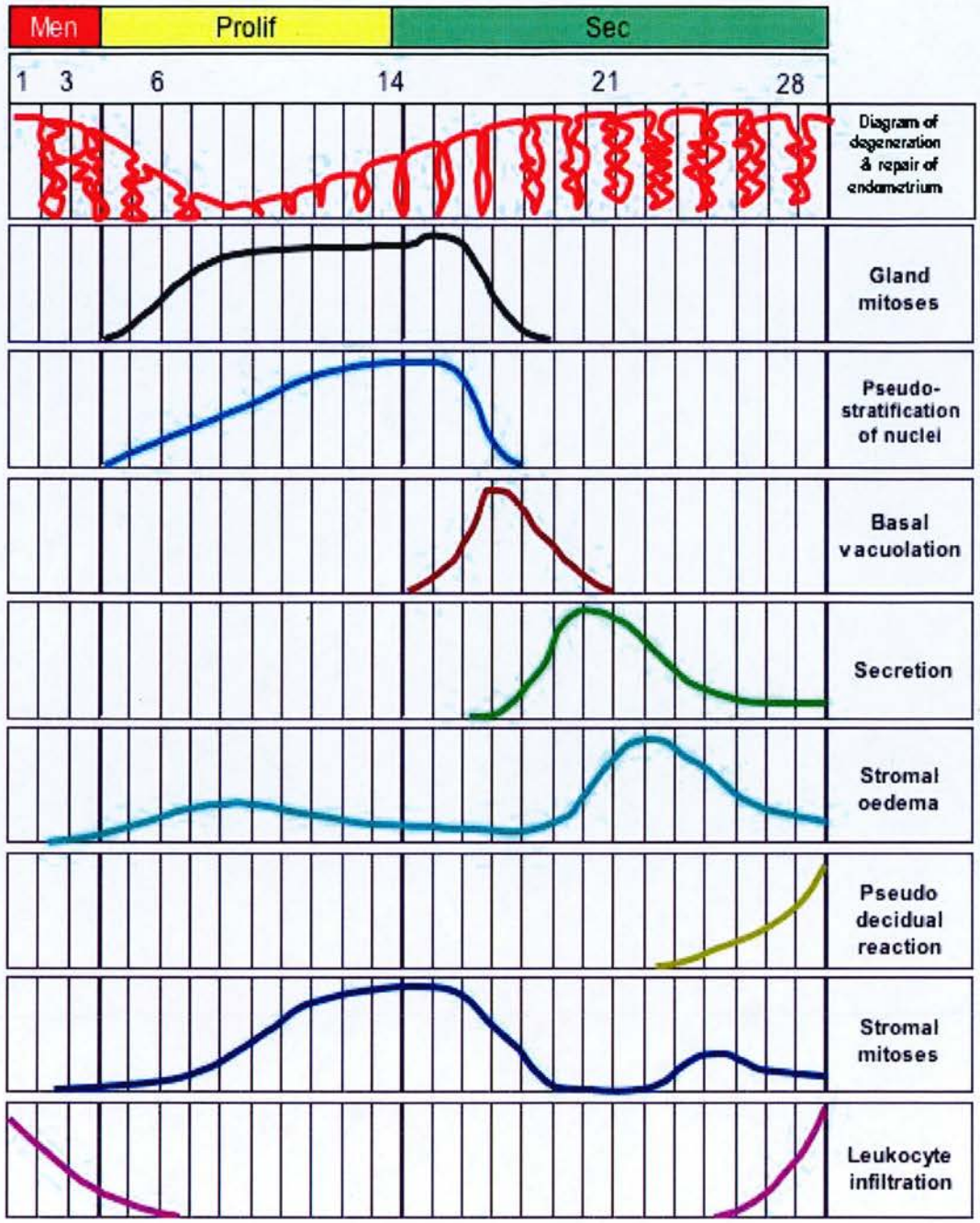


Figure 1.1: Diagrammatic representation of changes that take place in the human endometrium across the menstrual cycle, including the cyclical nature of degeneration and regeneration of the functional endometrial layer, based on the "classic" 28 day model as described by Noyes *et al* 1950, derived from (Noyes, Hertig et al. 1950). Men = Menstruation, Prolif = Proliferation, Sec = Secretory.

1.1.4 Decidualisation and the maintenance of pregnancy

As detailed in section 1.1.2, the transformation of endometrium to decidua begins in the secretory phase of the cycle under the influence of progesterone with characteristic changes observed in all compartments of the endometrium (Loke, King et al. 1995). First, secretion from the glandular epithelium is maximal during the implantation window with production of various hormonally regulated proteins including progesterone-associated endometrial protein (also known as glycodelin, placental protein 14, lactoglobulin, α-2 microglobulin, pregnancy-associated α-2 globulin) and albumin (Psychoyos 1992; Dockery 2002). In decidua, the glands in the upper two thirds of the tissue become atrophied and are non-secretory while those in the lower third continue secretory activity (Dockery 2002). Second, as detailed in section 1.1.2, the stroma also shows predecidual change that develops fully in the event of implantation. This stromal differentiation begins around post-ovulatory day 9 in oestrogen-primed stromal cells surrounding the spiral arterioles. Ultrastructurally, stromal decidualisation is characterised by progressive cell enlargement, rounding of the nucleus with coordinate increase in complexity and number of nucleoli, expansion of the secretory apparatus and dilation of the rough endoplasmic reticulum and golgi apparatus, in addition to cytoplasmic accumulation of glycogen (Wynn 1974; Cornillie, Lauweryns et al. 1985). This reaction then spreads throughout the stroma completing the pre-decidual transformation of the endometrium (Dunn, Kelly et al. 2003). In addition, extracellular matrix (ECM) proteins are distributed around each stromal cell with the decidual ECM being particularly rich in laminin and fibronectin. Decidualising stromal cells acquire the ability to secrete a variety of phenotypic antigens, including, insulin-like growth factor binding protein-1 (IGFBP-1) (also known as α1 pregnancy-associated endometrial protein, placental protein 12) and prolactin (Dunn, Kelly et al. 2003). Finally, increased coiling of the spiral arterioles begins in the secretory phase and continues in decidua. If pregnancy is established, these decidual changes become more widespread creating three layers: decidua compacta, decidua spongiosa and a basal undifferentiated layer, allowing regeneration following birth. uNK cells are the

main leukocyte population in first trimester decidua and these are further discussed in section 1.4.2.

The process of decidualisation is unique to species that undergo haemochorial placentation and is an essential pre-requisite for the establishment of a specialised environment for embryo implantation and trophoblast invasion (Finn 1986; Finn 1987; Bell 1990). Furthermore, among the species with haemochorial placentation, the degree of decidualisation appears to relate to the degree of trophoblast invasion (Ramsey, Houston et al. 1976; Christensen, Verhage et al. 1995).

1.1.4.1 Decidualisation Markers

Documented markers of the decidualisation process include prolactin (Maslar and Riddick 1979; Healy, Salamonsen et al. 1990; Tseng and Mazella 1999), IGFBP-1 (Bell, Jackson et al. 1991; Bryant-Greenwood, Rutanen et al. 1993; Lee, Giudice et al. 1997) and tissue factor (Lockwood, Krikun et al. 1994; Krikun, Schatz et al. 1998; Krikun, Schatz et al. 2000; Lockwood, Krikun et al. 2000), all of which have been used to confirm decidualisation induced *in vitro.* Other markers of decidualisation are reviewed in Tang *et al* (Tang, Guller et al. 1994). *In vitro* studies employing ESCs are commonly used as a model to characterise the molecular events regulating stromal decidualisation without the limitations of hormonal and cellular interactions that are inherently involved in the decidualisation process *in vivo*. These studies have elucidated many regulatory mechanisms involved in the decidualisation process, such as, altered steroid receptor expression and steroid metabolism, remodelling of ECM and cytoskeleton, altered expression of intracellular enzymes, growth factors and cytokines and their receptors, and induction of decidua-specific transcription factors (Oliver, Cowdrey et al. 1999; Popovici, Kao et al. 2000; Christian, Mak et al. 2002; Dunn, Kelly et al. 2003; Gellersen and Brosens 2003). However, the cellular interactions and target genes involved in decidualisation are complex and still poorly understood.

1.1.4.1.1 Prolactin

Prolactin (PRL) is synthesised within the anterior pituitary. Synthesis of PRL has been described in decidualised endometrial cells (Riddick, Luciano et al. 1978) and subsequently, it was confirmed that PRL production correlated with the extent of stromal decidualisation in a study of human endometrial explants from different stages of the menstrual cycle (Maslar and Riddick 1979). Furthermore, it has been demonstrated that increasing cell size associated with stromal decidualisation parallels the expression of PRL (Christian, Mak et al. 2002). PRL is considered to be important in human implantation and early pregnancy, as discussed in review by Jabbour and Critchley 2001 (Jabbour and Critchley 2001). Prolactin receptor knock-out female mice are sterile due to severely compromised preimplantation development and implantation failure of the few embryos reaching the blastocyst stage, strongly implicating PRL in control of reproductive functions, including implantation (Binart, Helloco et al. 2000). Prolactin is widely expressed and has been located in the pituitary lactotroph, dermal fibroblasts, epithelial cells of the prostate and breast, Leydig cells of the testis and in cells of haematopoetic lineage (Walters, Daly et al. 1983; DiMattia, Gellersen et al. 1988; Montgomery, Shen et al. 1992; Pellegrini, Lebrun et al. 1992; Ben-Jonathan, Mershon et al. 1996; Richards and Hartman 1996). In humans, PRL is a single copy gene consisting of five exons separated by four introns (Truong, Duez et al. 1984) (Figure 1.2). The pituitary transcription start site is located in exon 1 (Truong, Duez et al. 1984). Decidual PRL (dPRL) gene transcription requires activation of a tissue-specific promoter located approximately 5.7 kilobases (kb) upstream of the pituitary-specific promoter located in exon 1a (Berwaer, Martial et al. 1994; Gellersen, Kempf et al. 1994). Transcription from the decidual start site leads to an elongated 5'-untranslated region within the decidual mRNA transcript, but the open reading frames of the decidual and pituitary PRL mRNA results in identical proteins (Gellersen, DiMattia et al. 1989).

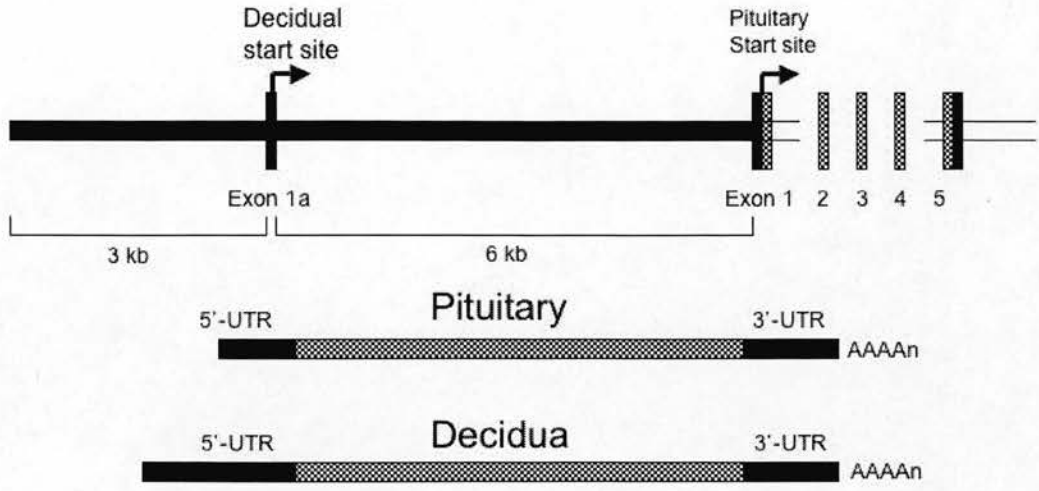


Figure 1.2: Human prolactin gene (above) and transcripts (below). Coding regions are depicted as chequered bars; non-coding regions are depicted as black bars. Initiation of transcription at exon 1a leads to an elongated 5'- untranslated region in the decidual transcript. Redrawn from Telgmann *et al*, 1998 (Telgmann and Gellersen 1998).

In the non-pregnant endometrium, dPRL is detected between the mid secretory phase and menstruation, coinciding with the first signs of decidualisation. dPRL protein is expressed primarily in stromal cells in the late secretory phase but epithelial cells are also immunoreactive for dPRL at this point in the cycle (Bryant-Greenwood, Rutanen et al. 1993). Whilst the protein has also been detected in both stromal and epithelial cells, mRNA has only been detected in the stromal cells (Dimitriadis, Salamonsen et al. 2000). In pregnancy, dPRL secretion progressively increases until a peak at 20 – 25 weeks, and declines toward term (Wu, Brooks et al. 1995). dPRL expression in the secretory endometrium is increased after *in vivo* administration of Medroxyprogesterone acetate (MPA) (Reis, Maia et al. 1999) and *in vivo* administration of the antiprogestin, mifepristone (RU486), significantly reduced dPRL expression in decidualised stromal cells (Wang, Zhu et al. 1994; Tseng and Mazella 1999). PRL mediates its effects via single pass transmembrane-spanning receptors that belong to the class 1 family of cytokine receptors. Two isoforms of the receptor have been described in humans (Kline, Roehrs et al. 1999). PRL receptor expression in the endometrium is temporally regulated throughout the menstrual cycle with minimal expression detected in glandular epithelial cells during the

proliferative phase with maximal expression observed in the mid-late secretory phase (Jabbour, Critchley et al. 1998; Jones, Critchley et al. 1998). In pregnancy, the receptor is maintained and localised to the decidua and foetal membranes (Maaskant, Bogic et al. 1996). A role for PRL in immune regulation is well documented (Yu-Lee 1997). PRL receptor expression has been reported in haematopoietic cells within the endometrium, including a novel target cell of PRL, the uNK cell (Jabbour, Critchley et al. 1998; Jones, Critchley et al. 1998; Gubbay, Critchley et al. 2002). PRL has also been shown to regulate the expression of interferon-regulatory factor (IRF-1), which is located in the glands and upregulated in the secretory phase (Jabbour, Critchley et al. 1999).

1.1.4.1.2 IGFBP-1

The insulin-like growth factor system consists of peptide growth factors (IGFs) and their receptors but also specific binding proteins (IGFBPs) that regulate the availability of IGFs to their receptors (Lamson, Giudice et al. 1991). Six types of soluble IGFBPs exist and each has a degree of tissue specificity (Shimasaki and Ling 1991). All six IGFBPs have been localised within the human endometrium and with the exception of IGFBP-5, all are seen to increase in the secretory phase compared with the proliferative stage (Zhou, Dsupin et al. 1994). The production of IGFBP-2 and –3 in cultures of ESCs treated with oestrogen and progesterone has been demonstrated (Giudice, Milkowski et al. 1991). Immunohistochemical studies have demonstrated an increase in IGFBP-1 levels in human endometrium in the secretory phase and early decidua (Bell and Drife 1989; Bryant-Greenwood, Rutanen et al. 1993). IGFBP-1 is considered a marker of decidualisation and its biosynthesis is used to assess this process in cultures of ESCs (Irwin, Kirk et al. 1989; Bell, Jackson et al. 1991; Giudice, Dsupin et al. 1992). The specific function of IGFBP-1 in the uterus in unknown although it may be regulating bioavailability of IGF-1, a stimulator of cell growth and PRL production in long term cultures (Rosenberg, Mazella et al. 1991). It has also been proposed to have a role in implantation (Giudice and Irwin 1999).

1.1.4.1.3 Tissue factor

Tissue factor (TF) is a membrane-bound glycoprotein, with an extracellular domain that acts as a receptor for factor VII, which is involved in the thrombin pathway and thought to maintain equilibrium between haemostatic and fibrinolytic pathways in the human endometrium (Schatz, Krikun et al. 2001). Indeed, TF is reported to augment VEGF production, further supporting TF's role in vascular haemostasis (Abe, Shoji et al. 1999). *In utero* haemorrhage in tissue factor knockout mice, has provided further evidence that TF is a major haemostatic agent, and implies a role for tissue factor in regulation of bleeding and murine blood vessel development (Carmeliet, Mackman et al. 1996). *In vitro* culture of ESCs has confirmed hormonal control of TF expression. After obtaining optimal TF levels, induced with oestrogen and progesterone treatment, a reduction of TF levels comparable to pre-treatment levels was observed with steroid hormone withdrawal (Lockwood, Nemerson et al. 1993). In decidualised ESCs, expression of TF parallels that of PRL (Lockwood, Nemerson et al. 1993) and is reported to be controlled by the specificity protein (SP)-1 transcription factor (Krikun, Schatz et al. 2000). *In vitro* studies have elucidated that progesterone stimulates SP-1 and antagonises SP-3, which in turn antagonises SP-1 production (Krikun, Schatz et al. 2000). Moreover, SP-1 is enhanced and SP-3 levels abrogated in the perivascular cells of secretory phase endometrium when progesterone levels are high (Krikun, Schatz et al. 2000). It has been proposed that the chronic upregulation of tissue factor in the decidualised stroma contributes to hemostasis during trophoblast invasion and remodelling of the vasculature (Nemerson 1988; Lockwood, Krikun et al. 1994). In the absence of pregnancy, TF levels are reported to fall prior to menstruation permitting menstrual-associated haemorrhage (Lockwood, Nemerson et al. 1993). In women using Norplant, a long-acting progestin-releasing contraceptive implant, the observed decline in endometrial TF protein and mRNA levels may partially account for their irregular bleeding patterns (Runic, Schatz et al. 1997; Runic, Schatz et al. 2000).

Other markers of decidualisation that have been reported in human and rat decidual stroma include vimentin, desmin, laminin and fibronectin (Glasser and Julian 1986;

Glasser, Lampelo et al. 1987; Van Muijen, Ruiter et al. 1987) consistent with structural rearrangements within the cells.

1.1.4.2 Progesterone and cAMP in decidualisation

Studies on ESC decidualisation *in vitro* have demonstrated that progesterone treatment alone is a weak inducer of the decidual transformation but can only stimulate cellular changes after prolonged exposure (Huang, Tseng et al. 1987; Zhu, Huang et al. 1990; Tseng, Gao et al. 1992; Mizuno, Tanaka et al. 1998). It has been reported that women with low serum progesterone levels at four weeks gestation can have successful pregnancies following assisted reproductive technologies (ART) treatment (Azuma, Calderon et al. 1993) therefore; it is possible that, in addition to progesterone, other local factors are important for decidualisation. A synthetic progestin, such as MPA, is usually used in place of progesterone for *in vitro* studies, as it is metabolically stable and thus more effective at inducing decidualisation and subsequent prolactin expression (Zhu, Huang et al. 1990 752). However, addition of cAMP in combination with MPA evokes decidualisation of ESCs in culture on a greatly reduced time scale (Tang, Guller et al. 1993; Tang, Guller et al. 1993; Brar, Frank et al. 1997) due to stimulation of the protein kinase A pathway (Brar, Frank et al. 1997). Evidence for the involvement of cAMP and its analogues in the decidualisation of human endometrium has been presented (Tang, Guller et al. 1993; Tang, Guller et al. 1993; Brosens, Takeda et al. 1996; Brar, Frank et al. 1997; Brosens, Hayashi et al. 1999) and experiments using cultures of ESCs have shown that prolactin expression is dependent on activation of the PKA pathway (Brar, Frank et al. 1997; Telgmann, Maronde et al. 1997) and treatment of ESCs with cAMP evokes an increase in prolactin mRNA expression and protein release (Tang, Guller et al. 1993; Brosens, Takeda et al. 1996; Brar, Frank et al. 1997; Brosens, Hayashi et al. 1999) which is sustained (Telgmann and Gellersen 1998). These authors demonstrated the ability of cAMP to synergise with a synthetic progestin to result in stromal differentiation and an increase in prolactin levels. High intracellular cAMP levels appear to act by sensitising the cells to progestins and thereby ensuring maximal prolactin expression (Telgmann and Gellersen 1998). Deletion of a region

of DNA in the dPRL promoter –332 bp to –270 bp relative to the transcription start site suppressed the ability of cAMP to induce prolactin expression identifying this particular region as an important target of cAMP induced signalling (Telgmann and Gellersen 1998).

It is thought that ligands such as PGE2, corticotrophin-releasing factor, and relaxin bind to their cognate receptors on the cell surface of ESCs and activate G-coupled proteins (Tang, Guller et al. 1993; Moy, Kimzey et al. 1996; Brar, Frank et al. 1997; Telgmann, Maronde et al. 1997; Christian, Marangos et al. 2001), which can be stimulatory (Gs) or inhibitory (Gi). The Gs proteins activate enzymes such as adenylate cyclase or phospholipase C, responsible for the generation of the second messengers, cAMP, and diacyglygerol (Meyer and Habener 1993). Two cAMP molecules can then bind to the latent PKA holoenzyme, comprised of two regulatory units (R) bound to two catalytic units (C), in the cytosol and induce a conformational change which dissociates C-subunits from the complex, activating PKA (Tasken, Andersson et al. 1994). The cAMP pathway is further discussed in section 1.6.3. The intracellular level of cAMP is not only regulated by the rate of synthesis but also its degradation. Phosphodiesterases (PDE) convert cAMP to AMP which no longer stimulates PKA activity, hence preventing augmentation of the progesterone-induced decidualisation process (Mehats, Andersen et al. 2002). It has been speculated that the increase in cAMP in decidualising cells may be attributable to inhibition of PDE activity (Gellersen and Brosens 2003), perhaps by such agents as relaxin (Bartsch, Bartlick et al. 2001; Ivell 2002). In cultures of human ESCs, interaction of relaxin with LGR7 and LGR8, the relaxin receptors (Hsu, Nakabayashi et al. 2002; Sudo, Kumagai et al. 2003), both acutely and permanently elevates intracellular cAMP levels in concert with induction of prolactin secretion (Telgmann and Gellersen 1998) and produces maximal stimulus on prolactin production when added in combination with a synthetic progestin (Huang, Tseng et al. 1987; Zhu, Huang et al. 1990).

The mechanisms and factors involved in decidualisation are complex and it is likely that other factors that are involved have not yet been identified.

1.1.4.3 Implantation

Successful implantation requires the simultaneous development of the endometrium to a receptive state and the embryo to blastocyst stage. The endometrium is receptive to implantation only for a few days in each menstrual cycle (the implantation window) (Navot, Anderson et al. 1989; Li, Warren et al. 1991; Tabibzadeh 1998). The implantation window falls in the mid-late secretory phase, day 20 and 24 in a "28 day" cycle, in the human (Hertig, Rock et al. 1956; Bergh and Navot 1992; Harper 1992) (section 1.1.2).

The implantation process itself is quite unique in humans in comparison to other species and is haemochorial (Pijnenborg, Robertson et al. 1981). After fertilisation of the oocyte within the fallopian tube, the zygote undergoes successive cleavage events forming a morula which enters the uterine lumen around 72-96 hours post fertilisation (Bulletti, Polli et al. 1994; Edwards 1994). The blastocyst forms on day 5 and comprises an inner cell mass with an outer layer of trophectoderm (Edwards 1994). Subsequently, the peripheral cytotrophoblast differentiates into syncytiotrophoblast. Human chorionic gonadotrophin begins to be produced by the embryo preventing the demise of the corpus luteum and is further discussed in section 1.1.4.4.

Implantation occurs in three stages: apposition, adhesion, and invasion (Bulletti, Polli et al. 1994; Edwards 1994). The apposition stage occurs when the hatched blastocyst is present in the uterine lumen and in close, but not direct, contact with the luminal epithelium (Bulletti, Polli et al. 1994; Edwards 1994). Between days 6-7 postfertilisation, adhesion of the blastocyst to the uterine epithelium occurs involving direct interaction of the trophectoderm with the epithelium (Bulletti, Polli et al. 1994; Edwards 1994). After this attachment, the trophoblast invades the endometrium. Placenta formation begins once the blastocyst has invaded and become embedded in the decidua (Bulletti, Polli et al. 1994; Edwards 1994). Several cytotrophoblast cells extend through the syncytiotrophoblast and invade the decidua where they fuse forming the cytotrophoblast shell, which surrounds the inner surface of the

implantation site, anchoring the developing trophoblast to the maternal decidua (Bulletti, Polli et al. 1994; Edwards 1994). Villous trophoblast cells will eventually form a layer over the placenta and have the function of nutrient and oxygen transport from maternal blood to the foetus (Bulletti, Polli et al. 1994). The extravillous trophoblasts also invade the decidual spiral arterioles and become incorporated into the vascular wall, changing its plasticity and allowing the delivery of greater volumes of blood (Pijnenborg, Robertson et al. 1981; Aplin 1991; Edwards 1995; Tabibzadeh and Babaknia 1995). In cases of under-invasion of the trophoblast, pathologies such as miscarriage, pre-eclampsia and foetal intrauterine growth restriction (IUGR) can result (Loke and King 1995). Such foetal conditions can lead to the development of problems in adult life (Barker 1997; Barker 1997; Barker 1997). Conversely, when over-invasion occurs there is abnormal placentation and a "placenta percreta" can develop (Bjercke 1999).

Implantation clearly involves a tightly orchestrated series of events which requires the controlled expression of a variety of molecules (Psychoyos 1986; Simón, Francees et al. 1995; Simon, Pellicer et al. 1995; Tabibzadeh and Babaknia 1995; Giudice 1999; Giudice 1999; Aplin 2000). Therefore, it is important for this process to be under stringent control and a synchrony between the developing blastocyst and the endometrium is fundamental for successful implantation. This is likely to occur via the actions of steroid hormones, oestradiol and progesterone, and soluble mediators such as colony-stimulating-factor (CSF) (Kauma, Aukerman et al. 1991; Pollard, Hunt et al. 1991; Daiter, Pampfer et al. 1992), leukaemia inhibitory factor (LIF) (Charnock-Jones, Sharkey et al. 1994; Stewart 1994; King, Jokhi et al. 1995; Sawai, Azuma et al. 1995; Nachtigall, Kliman et al. 1996), and integrins (Lessey, Damjanovich et al. 1992; Tabibzadeh 1992; Lessey, Castelbaum et al. 1994), to name but a few of the local factors involved in an extremely complex process.

Imbalances of the steroid hormones, oestrogen and progesterone, and/or their receptors can result in an "out-of-phase" development that has the potential to be significantly detrimental to a successful implantation (Psychoyos 1986; Bonhoff, Johannisson et al. 1990). As both processes are steroid hormone-dependent,

implantation and decidualisation are likely to be closely linked, especially as one important feature of a receptive endometrium is pre-decidualisation of the stroma. In humans, decidualisation is a blastocyst-independent event, but successful blastocyst implantation is a decidualisation-dependent event (Rosenwaks 1987; Sarani, Ghaffari-Novin et al. 1999). In contrast, implantation has been shown to be dependent on embryonic age rather than endometrial maturation in another study involving IVF treatment of women (Bergh and Navot 1992). The receptive endometrium is thought to balance implantation with excessive trophoblast invasion by providing appropriate signals (Tabibzadeh and Babaknia 1995) essential for the implantation process and maternal acceptance (Edwards 1994).

For successful implantation, stringently regulated interactions between the maternal decidua and the foetal trophoblast must occur (Aplin 2000; Aplin and Kimber 2004; Staun-Ram and Shalev 2005). The decidua secretes products e.g. IGFBP-1, that have been demonstrated, in an *in vitro* model, to bind to trophoblast-specific integrins in order to modulate trophoblast migration, invasion or both (Irving and Lala 1995; Hamilton, Lysiak et al. 1998; Giudice 2003). In turn, the invading trophoblast secretes matrix metalloproteinases (MMPs) (discussed further in section 1.3.3) that act to breakdown the decidual extracellular matrix, further facilitating remodelling of the decidua to permit trophoblast invasion. To limit overinvasion of the maternal decidua, the decidual stromal cells express high levels of the tissue inhibitors of MMP (TIMPs) (discussed further in section 1.3.3), which have also been demonstrated to modulate trophoblast invasiveness *in vitro* (Aplin and Kimber 2004). Cytokines such as IL-1 and TGFβ (discussed further in section 1.5.1.2 and 1.5.1.12 respectively), present at the decidual – trophoblast interface, are reported to regulate trophoblast invasion *in vitro* by stimulating MMP and TIMP production, respectively (Staun-Ram and Shalev 2005). Trophoblast-derived cytokines, such as insulin-like growth factor 2, inhibit IGFBP-1 expression in human decidualised stromal cells *in vitro* (Hamilton, Lysiak et al. 1998), providing evidence to suggest that trophoblast invasion and migration is self-controlled in addition to control by the maternal microenvironment through molecular and cellular interaction (Aplin 2000; Aplin and Kimber 2004; Staun-Ram and Shalev 2005).

1.1.4.4 Human chorionic gonadotrophin (hCG)

The oncofoetal antigen, hCG, is a heterodimeric glycoprotein hormone secreted by the syncytiotrophoblast cells in the chorionic villi (Rao 2001). hCG is composed of 244 amino acids with a molecular mass of 36.7 kDa (Rao 2001). hCG is composed of noncovalently associated α and β subunits and multiple N-linked and O-linked oligosaccharide side chains (Rao 2001). The α subunit is identical to that of luteinising hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH) but the β subunit is unique to hCG (Rao 2001). hCG is detectable in maternal serum as early as 1 day after the initiation of embryo implantation and rapidly escalates to a peak value of more than 100,000 mIU/ml around 60 – 90 d gestation before gradually declining to levels under 50,000 mIU/ml within the second trimester (Jaffe, Lee et al. 1969). These values vary significantly between individual patients. The primary role of hCG is to prevent demise of the corpus luteum (CL) and maintain luteal progesterone production thereby ensuring embryo survival (Yen, Jaffe et al. 1999). In addition, hCG is an established tumour-associated antigen that is over expressed in a variety of cancers, including colon, lung, pancreas, oesophagus, breast, bladder, cervix, stomach, and prostate (Triozzi and Stevens 1999). The hormonal actions of hCG are propagated via the LH/hCG receptor, which is the same g-protein coupled receptor that can be activated by both proteins (Rao 2001). The LH/hCG receptor was originally reported to be expressed on ovarian granulosa cells, theca interna cells and luteal cells but has subsequently been located in the human endometrium (Reshef, Lei et al. 1990; Lei, Reshef et al. 1992) and myometrium (Kornyei, Lei et al. 1993). Owing to its receptor's endometrial locus, hCG has been implicated in full decidualisation of ESC and in support of this, human endometrial stromal cells, cultured *in vitro*, decidualise in response to hCG treatment (Han, Lei et al. 1999; Su, Mi et al. 2002). In addition, the mRNA for the LH/hCG receptor has also been detected in T lymphocytes derived from the peripheral blood of pregnant women (Lin, Lojun et al. 1995).

1.2 Oestrogen and Progesterone

The steroid hormones, including progestins, oestrogens, androgens, glucocorticoids and mineralocorticoids, are small, potent, lipophilic molecules that regulate a wide array of cellular biological and physiological functions, such as proliferation, survival, differentiation, organ development, metabolism, and haemostasis. In the female reproductive tract, the coordinated actions of the steroid hormones, oestrogen and progesterone (Figure 1.3), are pivotal to succinct female reproductive functions. They act throughout the reproductive tract, including the hypothalamus, pituitary, uterus, and ovary. Oestrogens act on the uterus to increase cellular proliferation across the proliferative phase and also stimulate expression of progesterone receptors (PR) during this phase. The presence of a functioning PR is crucial to fertility in mice with the PR null mouse being infertile displaying defective ovulation, implantation, and stromal cell decidualisation (Conneely, Mulac-Jericevic et al. 2002). The actions of oestrogen and progesterone are mediated via their cognate receptors both of which are expressed in the human endometrium (section 1.2.2).

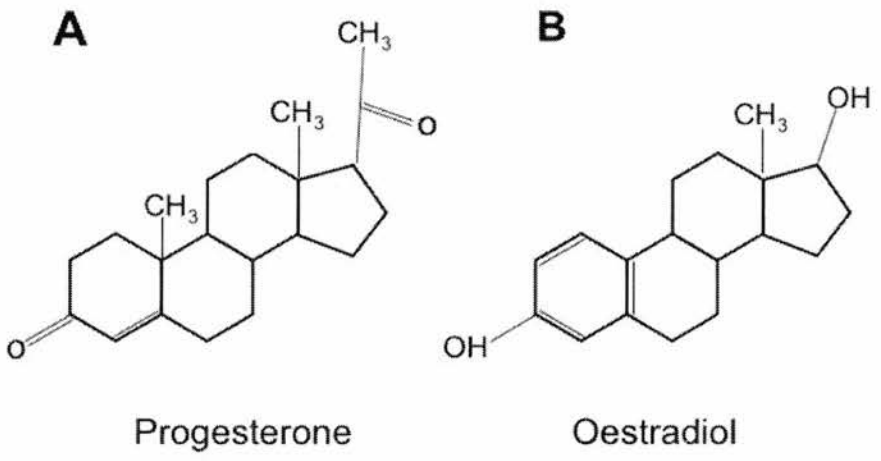


Figure 1.3: Diagram depicting the structure of; A, progesterone and B, oestradiol.

1.2.1 Structure and function of steroid receptors

The actions of oestrogen and progesterone are mediated via their binding to specific intracellular receptors, located primarily in the nuclear compartment. Both oestrogen and progesterone receptors are members of the nuclear receptor superfamily of transcription factors which encompasses the receptors for steroid hormones, thyroid hormones, vitamin D and retinoic acid, as well as several "orphan" receptors (Robinson-Rechavi, Escriva Garcia et al. 2003). The nuclear steroid receptors share common structure and functional domains that can be exchanged between related receptors without loss of function, as summarised in Figure 1.4. AF-1 and AF-2 are two transcriptional activation functional domains that mediate the transactivational activity of all steroid hormone receptors. AF-1 is located in the N-terminal region and contributes to constitutive ligand-independent activation by the receptor. AF-2 is a short amphipathic α-helix, ligand-dependent transcriptional activation functional domain, located in the C terminal of the receptor and is highly conserved between members of the nuclear receptor superfamily. Steroid receptors are comprised of five regions, denoted A/B, C, D, E and F. The A/B region is located at the N-terminus and contains the AF-1 domain. The C region contains a highly conserved DNA-binding domain (DBD), spanning 60 -70 residues. This domain contains amino acids required for the recognition of the specific hormone response elements and dimerisation. Aberrations in DNA and mutations of the C region can result in receptor dysfunction. Proximal to the C region is the hinge or D region, which acts as a bridge between the DBD and the ligand-binding domain (LBD). This enables the receptor to adopt different conformations by rotation. Region E or the LBD accommodates the transactivation domain, AF-2. This region, as the name suggests, is responsible for the ligand-dependent activation of the receptor. The LBD is also responsible for determining whether or not the receptor is activated, interaction with heat shock proteins, mediation of homo and heterodimerisation and transcriptional repression function (Gronemeyer and Laudet 1995; Chambon 1996; Moras and Gronemeyer 1998). Region F is present in the C-terminal of the LBD in some steroid nuclear

receptors. The region is not well conserved and to date, no function identified.

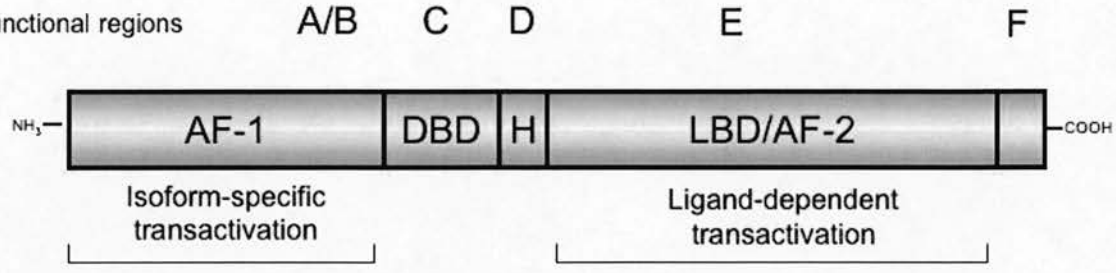


Figure 1.4: Functional domains of nuclear/steroid receptors. The functional and structural regions A/B, C, D, E and F are indicated in the schematic diagram. Also shown are the two transcription activation function regions (AF-1 and AF-2). Adapted from Brosens *et al*, 2004 (Brosens, Tullet et al. 2004).

In the absence of ligand, the receptors are inactive and are present in complexes containing other proteins such as heat shock protein 90. Hormone-bound receptors are recruited to target gene promoters via homodimer binding to specific DNA sequences in target genes known as hormone response elements (Carson-Jurica, Schrader et al. 1990). The PR and the glucocorticoid receptor (GR) bind to the same consensus sequences (glucocorticoid/progesterone response element; GRE/PRE) and it is thought that, in the uterus, progesterone has local immunosuppressive effects similar to the systemic effects of the glucocorticoids (Brosens, Tullet et al. 2004). Once tethered to the promoter regions in target genes the steroid receptors can modulate gene transcription resulting in a function change. In addition, nonconsensus sequences (nGRE) exist which allow binding of the PR or GR to suppress gene transcription. Steroid hormone actions may also be mediated via membrane receptors, in addition to nuclear receptors, and may interact with intracellular second messengers or other signal transduction pathways in a non-genomic manner (Boonyaratanakornkit and Edwards 2004). It should also be noted that, when present in high concentrations, steroid hormones are able to mediate their actions act via non-genomic mechanisms e.g. modulation of ion channel function (for review see Revelli *et al* (Revelli, Massobrio et al. 1998)).

1.2.2 Oestrogen and Progesterone receptors

The progesterone receptor was first identified in the chick oviduct and mammalian uterus in the 1970s (Milgrom, Atger et al. 1970; Sherman, Corvol et al. 1970; Leavitt and Blaha 1972). Two isoforms of the progesterone receptor (PR) exist, PR A and PR B (Horwitz and Alexander 1983; Lessey, Alexander et al. 1983). PR A and PR B are derived from different promoter usage of the same gene located on chromosome 11 and function as transcriptional regulators of progestin-responsive genes (Clarke, Roman et al. 1990; Kastner, Krust et al. 1990). PR A differs from PR B in that it is devoid of 164 amino acids at the N-terminus (B-upstream sequence, BUS) (Horwitz and Alexander 1983). Both forms of PR are activated by progesterone but they are thought to have differing functions. PR B is the more transcriptionally active isoform (Wen, Xu et al. 1994) and in settings where both the A and B forms are present PR A can act as a dominant repressor of PR B function (Tung, Mohamed et al. 1993; Vegeto, Shahbaz et al. 1993). This suggests that in cells where there is an abundance of PR A the responsiveness to progesterone may be reduced and that the ratio of PR A: PR B may determine the cellular response to the hormone. PR A has also been shown to inhibit the function of the glucocorticoid, mineralocorticoid, androgen and oestrogen receptors (McDonnell, Clemm et al. 1994; McDonnell and Goldman 1994; Wen, Xu et al. 1994). A third PR, PR-C, has been identified and has been speculated to be capable of altering the transcriptional activity of PR A and PR B (Wei, Gonzalez-Aller et al. 1990; Wei, Hawkins et al. 1996). Additionally, a novel membrane protein unrelated to nuclear PR was recently identified (Losel and Wehling 2003; Zhu, Bond et al. 2003). It is reported to have the properties of a G-protein-coupled receptor for progesterone and has been shown to be involved in mediating the extranuclear signalling actions of progesterone that promotes oocyte maturation in fish. The role of this membrane PR (mPR) in mammalian cells is unknown. One possible suggestion is the regulation of rapid non-transcriptional signalling of progestins via the relationship between the membrane and classical nuclear PR, although this has not yet been explored. The functional importance of this receptor with context to the endometrium is currently under investigation

(Fernandes, Pierron et al. 2005; Karteris, Zervou et al. 2006; Krietsch, Fernandes et al. 2006).

There are two structurally related isoforms of oestrogen receptor (ER), namely, ERα (Green, Kumar et al. 1986) and ERβ (Kuiper, Enmark et al. 1996; Mosselman, Polman et al. 1996). *In vitro* both proteins are functional and both bind oestradiol with a high affinity (Mosselman, Polman et al. 1996).

1.2.3 Co-regulators

In addition to regulating gene expression via direct interaction with the general transcription machinery, steroid hormone receptor function is modulated by the recruitment of co-activators and co-repressors. Examples include the p160 or steroid receptor coactivator (SRC) family (Lee, Lee et al. 2001), CREB-binding protein (CBP), the CBP-related factors p300 and p/CAF (p300/CBP-associated factor) (Chen and Li 1998; Lee, Lee et al. 2001). Co-regulators either possess chromatin-remodelling activity or facilitate interaction between steroid receptors and the transcription machinery (Brosens, Tullet et al. 2004).

As previously mentioned, two isoforms of the PR exist. Whilst the two forms of PR have similar DNA- and ligand-binding affinities (Christensen, Estes et al. 1991), they have opposite transcriptional activities (Tung, Mohamed et al. 1993; Vegeto, Shahbaz et al. 1993; Chalbos and Galtier 1994; McDonnell, Clemm et al. 1994; Zamir, Harding et al. 1996). In most contexts, PR B functions as an activator of progesterone-responsive genes, while PR A is transcriptionally inactive (Tung, Mohamed et al. 1993; Vegeto, Shahbaz et al. 1993). In addition, PR A also functions as a strong transdominant repressor of PR B (Vegeto, Shahbaz et al. 1993) and human oestrogen receptor (ER) transcriptional activity in the presence of both PR agonists and antagonists (Vegeto, Shahbaz et al. 1993; McDonnell and Goldman 1994; Zamir, Harding et al. 1996; Giangrande, Pollio et al. 1997). The lower transactivation potential of PR A may be explained by its higher affinity for the co-repressor SMPT

(silencing mediator of retinoid and thyroid hormone receptor) and its less efficient recruitment of the co-activator SRC-1 (Giangrande, Kimbrel et al. 2000).

1.2.4 Oestrogen and progesterone receptor localisation across the normal menstrual cycle

Several immunohistological studies within the human endometrium have described the distribution and cyclical variation of oestrogen and progesterone receptor expression within the epithelial and stromal compartments in both the functional and basal layers of endometrium (Garcia, Bouchard et al. 1988; Lessey, Killam et al. 1988; Snijders, de Goeij et al. 1992; Critchley, Bailey et al. 1993; Snijders, de Goeij et al. 1996; Wang, Critchley et al. 1998; Critchley, Kelly et al. 2001; Slayden, Nayak et al. 2001). These studies report that both receptors are immunolocalised within cell nuclei. Previous studies have reported that PR isoforms levels change throughout the normal menstrual cycle with the PR A: PR B ratio greater premenstrually and immediately after menstruation (Mangal, Wiehle et al. 1997). In addition, the PR B isoform is differentially regulated in endometrial stroma (Wang, Critchley et al. 1998). PR A and PR B are located in the glands and stroma during the proliferative phase. During the secretory phase, PR A and PR B expression decreases in the epithelium but the PR A isoform persists until the late secretory phase in the stromal cells, particularly those in the perivascular region (Wang, Critchley et al. 1998). PR B is reported to be present in the stromal cells until the mid secretory phase and it is suggested that this isoform may be responsible for the progestogenic effects which cause glandular secretion (Mote, Balleine et al. 1999). These immunolocalisation studies provide circumstantial evidence to imply that PR A is the isoform responsible for the secretory phase actions of progesterone within the stroma, with particular relevance to the initiation of decidualisation. In addition, the localisation of both PR isoforms to the predecidual cells around the spiral arterioles suggests that progesterone withdrawal effects may be profound in these cells (Wang, Critchley et al. 1998).

In vitro studies have demonstrated that PR A is more abundant that PR B during the process of decidualisation (Brosens, Hayashi et al. 1999). Interestingly, Brosens *et al*

also report that PR A was augmented with the progression of decidualisation and that this reduction was accelerated with addition of MPA, a synthetic progestin (Brosens, Hayashi et al. 1999). In addition, transient transfection of either PR A or PR B promoter-reporter constructs resulted in inhibition of dPRL in response to cAMP. Exposure of the endometrium to locally delivered high dose progestins, as with the Levonorgestrel-releasing intrauterine system (LNG-IUS), down-regulates ERα, PR A and PR B in both glandular and stromal cells (Critchley, Wang et al. 1998). The administration of RU486, a PR antagonist, in the early secretory phase of the cycle prevents progesterone-induced down regulation of PR and ERα, confirming the role of progesterone in mediating uterine functions (Maentausta, Svalander et al. 1993; Cameron, Critchley et al. 1996).

Both ERα and ERβ have been detected in glandular epithelium, stroma and perivascular cells. Immunoexpression of ERα increases in both endometrial epithelium and stroma of the functionalis throughout the proliferative phase and reaches maximal expression in the late proliferative endometrium, at the time of ovulation. Levels then decline in the glandular and stromal compartments of the functional layer during the secretory phase (Critchley 2000). *In situ* hybridisation has detailed the localisation of mRNA for both ER isoforms within the human endometrium (Matsuzaki, Fukaya et al. 1999) and found an identical pattern of expression to that of the ERα protein. ERβ mRNA was expressed predominantly by the glandular epithelium with only faint stromal staining (Matsuzaki, Fukaya et al. 1999). Again, decreased expression was apparent in the functionalis of secretory phase endometrium (Matsuzaki, Fukaya et al. 1999; Critchley, Brenner et al. 2001; Lecce, Meduri et al. 2001).

1.3 Mechanisms of Menstruation

The physiological withdrawal of progesterone from an oestrogen-progesterone primed endometrium is the triggering event for the cascade of molecular and cellular interactions that result in menstrual bleeding. Several key inflammatory mediators, many of which have a key perivascular location, are involved in the current hypothesis of the causes of menstruation, which is based on lines of evidence derived

from studies on local endometrial response to progesterone withdrawal (Critchley, Kelly et al. 2001).

1.3.1 Prostaglandins

The vascular changes found to be associated with menstruation have implicated the vasoactive substances present in the endometrium, PGE_2 and $PGF_{2\alpha}$ (Lumsden, Kelly et al. 1983; Lumsden, Brown et al. 1984) as key regulatory molecules. Prostaglandins (PGs) are lipid mediators that are produced from arachidonic acid (AA). Two rate-limiting steps, phospholipase A2 activity, and activity of the two Cyclooxgenase enzymes, COX-1 and 2, control the production of prostaglandins (PGs). Free AA is initially converted to prostaglandin H_2 (PGH_2) by COX-1 and –2. Subsequently, the action of specific synthases on PGH_2 directs the synthesis of the five primary prostanoids, namely PGD_2, PGE_2, $PGF_{2\alpha}$ PGI_2 and TXA_2 (Smith 1986; Smith and Dewitt 1996; Smith, Garavito et al. 1996). PGs were first implicated in the process of menstruation approximately 40 years ago (Pickles 1967). Subsequently, increases in PG synthesis and decreases in metabolism have been reported in response to falling progesterone levels (Ylikorkala and Makila 1985). Furthermore, the vasoconstrictive actions of $PGF_{2\alpha}$ have been implicated in the initiation of menstruation (Baird, Cameron et al. 1996). The ratio of PGE_2: $PGF_{2\alpha}$ is reported to be increased in the menstrual fluid of women suffering from excessive menstrual bleeding (Rees, Anderson et al. 1984; Cameron, Leask et al. 1987) and administration of nonsteroidal anti-inflammatory drugs (COX inhibitors) in menstrual pathologies reduces blood loss suggesting that aberrant expression of the vasodilator PGE_2 promotes increased bleeding which can lead to menorrhagia (Fraser 1992).

In vitro tissue explant culture studies suggest that the endometrium has a greater capacity to produce PGs in the progesterone-dominant secretory phase of the menstrual cycle (Abel and Baird 1980). Furthermore, the PG metabolising enzyme, prostaglandin dehydrogenase (PGDH), has been found to be under hormonal control (Casey, Hemsell et al. 1980; Greenland, Jantke et al. 2000). Within the human endometrium, PGDH is located within the cytoplasm of gland cells and levels were

found to be highest in the secretory phase but lowest premenstrually and during menstruation, consistent with the *in vitro* culture data (Casey, Hemsell et al. 1980). This is in agreement with a study by Critchley et al who also report a significant increase in PGDH levels in secretory phase endometrium (Critchley, Wang et al. 1998). In addition, these authors also demonstrated that PGDH activity is significantly increased at 12 months post-insertion of a LNG-IUS and coincides with a rise in PR A and PR B immunostaining, implicating both receptor levels and PGs in the control of endometrial bleeding (Critchley, Wang et al. 1998). COX-2 expression and immunoreactivity levels, while present in the glandular epithelium throughout the menstrual cycle, are greatest in the perivascular region upon premenstrual progesterone withdrawal coinciding with the recruitment of leukocytes into the endometrium immediately prior to menstruation (Jones, Kelly et al. 1997; Critchley, Jones et al. 1999).

Moreover, the actions of PGs on the blood vessels and perivascular cells are underlined by the significant distribution of PG receptors in this locus (Milne, Perchick et al. 2001; Milne and Jabbour 2003). There are 4 different PGE_2 receptors and these are referred to as EP_{1-4}. The EP_2 and EP_4 receptors act via G_s proteins to induce an increase in cAMP levels. The EP_3 receptor exists as 7 splice variants (Adam, Boie et al. 1994; Sugimoto, Narumiya et al. 2000) with the predominant effect of decreasing cAMP levels. EP_1 acts via a different second messenger, Ca^{2+}, to exert its effects (Kennedy, Coleman et al. 1982; Watabe, Sugimoto et al. 1993).

1.3.2 Vascular Endothelial Growth Factor (VEGF) and Hypoxia

Coincident vasoconstriction of the endometrial spiral arteries (Markee 1940) and vasoconstriction and contractions of the myometrium in response to increased production of $PGF_{2\alpha}$ (Ylikorkala and Makila 1985) are presumed to induce hypoxia within the uppermost endometrial zone. Hypoxia is a potent inducer of angiogenic and vascular permeability factors such as vascular endothelial growth factor (VEGF) (Popovici, Irwin et al. 1999; Zhang and Salamonsen 2002). VEGF is expressed across the menstrual cycle (Zhang, Scott et al. 1998) and is upregulated premenstrually in response to the aforementioned hypoxic stimulus (Sharkey, Day et

al. 2000). A recent microarray analysis has demonstrated that *in vitro* decidualised ESCs express greater levels of VEGF as compared with their non-decidualised counterparts (Popovici, Kao et al. 2000), implying a role for stromal decidualisation in increasing VEGF levels. This is further supported by the observation that relaxin, a decidualisation stimulus, increases VEGF expression in ESC *in vitro* (Unemori, Erikson et al. 1999). The type 2 receptor for VEGF, kinase domain receptor (KDR), is expressed only in endometrial endothelial cells until the premenstrual phase when progesterone withdrawal upregulates expression of both KDR and VEGF in the superficial stroma in both women and nonhuman primates (Nayak, Critchley et al. 2000; Nayak and Brenner 2002). Pro-MMP-1 is also induced in a coordinate manner in the same stromal population in response to progesterone withdrawal (Nayak and Brenner 2002). Hence, VEGF, KDR and MMPs are coordinately expressed by stromal cells in the upper functionalis at the time of progesterone withdrawal, intimating that they may act in concert to regulate the menstrual process.

1.3.3 Matrix metalloproteinases

At present, there is no certainty as to the source of the matrix metalloproteinases (MMPs) which are enzymes thought to be released by both the invading immune cells and by the stromal cells. There are several major subgroups of MMPs: the collagenases (e.g. MMP-1), the gelatinases (MMP-2 and 9), the stromelysins (MMP-3, 10 and 11) and the membrane-type MMPs. MMPs are secreted as inactive zymogens, which are activated by proteases. The degeneration of the extracellular matrix has been attributed to the actions of MMPs (Salamonsen 1996; Schatz, Papp et al. 1997; Lockwood, Krikun et al. 1998; Salamonsen and Woolley 1999) and their role in menstruation implied by the timing of their expression within the human endometrium (Rodgers, Osteen et al. 1993; Hampton and Salamonsen 1994; Rodgers, Matrisian et al. 1994). Additionally, progesterone withdrawal has been found to increase MMP release in *in vitro* culture of endometrial stromal cells (Salamonsen, Butt et al. 1997; Lockwood, Krikun et al. 1998). Withdrawal of progesterone from cultures of endometrial stromal cells (ESCs) enhances proMMP-2 (Irwin, Kirk et al. 1996), proMMP-3 (stromelysin-1) production (Schatz, Papp et al. 1994) and upregulation of MMP-1, -2, -3 and -4 in latent form (Salamonsen, Butt et al. 1997).

The effects of progesterone on MMPs may be attributable to the progesterone-stimulating enhancement of tissue inhibitors of MMPs (TIMPs) synthesis, such as TIMP-3 (Higuchi, Kanzaki et al. 1995). TIMPs are endogenous inhibitors that act by forming 1:1 complexes with the MMP. In addition, it has also been demonstrated that progesterone inhibits MMP production by acting synergistically with TGFβ1 (Bruner, Eisenberg et al. 1999; Yuan and Varga 2001; Jabbour, Kelly et al. 2006). Although MMPs are clearly involved in the tissue breakdown involved in menstruation their activation is likely to be several steps downstream of the initial vasoconstriction events reported by Markee (Markee 1940).

1.3.4 Endothelins

Studies on the role of vasoactive mediators in menstruation have investigated a group of 21 amino acid peptides, the endothelins (Marsh, Findlay et al. 1996). They are cleaved from precursor (pro- and preproendothelins) proteins and to date, three endothelins have been identified, endothelin-1, 2 and 3. Endothelin-1 (ET-1) is reported to be the most potent vasoconstrictor identified. ETs signal via two G-protein coupled receptors, ET_A and ET_B, it has been suggested that the endothelins may act via the ET_B receptor to cause vasoconstriction during menstruation (Collett, Kohnen et al. 1996; Wang, Zheng et al. 1998). All three isoforms have been localised to the glandular and luminal epithelium (O'Reilly, Charnock-Jones et al. 1992; Salamonsen, Butt et al. 1992; Cameron, Plumpton et al. 1993) with ET-1 also present in the stroma and in some endothelial cells (Salamonsen, Butt et al. 1992; Ohbuchi, Nagai et al. 1995) across the menstrual cycle in human endometrium. ET-1 protein (Ohbuchi, Nagai et al. 1995) and mRNA (Economos, MacDonald et al. 1992) levels have been found to increase in the premenstrual-menstrual phase suggesting a role in menstruation (Salamonsen, Marsh et al. 1999). This is further supported by ET-1 release in response to progesterone withdrawal from endothelial cells isolated from human small uterine arteries (UtMVECs), serving to explain the vasoconstriction seen in the coiled arteries during menstruation in humans (Edlund, Andersson et al. 2004). It has been reported that TGFβ1 dose-dependently increased ET-1 release from 1st trimester decidual cells (Kubota, Taguchi et al. 1997). This may be explained by TGFβ1 opposing the progesterone-induced increase in enkephalinase expression,

the enzyme responsible for degradation of ET (Fagny, Michel et al. 1991), in endometrial stromal cells, even in the continued presence of MPA (Casey and MacDonald 1996). In addition ET-1 has been reported to induce $PGF_{2\alpha}$ release from human endometrium (Cameron, Davenport et al. 1991). Together these studies have highlighted ET-1 as a potent vasoconstrictor, involved in endometrial bleeding prior to the onset of menstruation and, subsequently, in cessation of menstrual bleeding (Salamonsen, Marsh et al. 1999).

1.4 Menstruation and Inflammation

In addition to hormonal control, menstruation is considered to be an inflammatory event with many inflammatory mediators upregulated and localised to a perivascular location (Finn 1986; Critchley, Kelly et al. 1994; Kelly 1994; Jones, Kelly et al. 1997; Critchley, Jones et al. 1999; Milne, Critchley et al. 1999; Salamonsen 2003). Inflammation is characterised by tissue oedema, recruitment of inflammatory cells to the area and the associated release of pro-inflammatory cytokines (Salamonsen 2003). Leukocytes invade the endometrium during the pre-menstrual phase, coinciding with the withdrawal of progesterone. Indeed an elegant study in sheep demonstrated that leukocytes migrate into the endometrium, but not the myometrium, following ovarian progesterone withdrawal (Staples, Heap et al. 1983). In addition to providing defence against pathogens, when activated, leucocytes produce a plethora of regulatory molecules, including cytokines, chemokines and a range of enzymes that are important either directly in matrix degradation, or indirectly by activation of other proteases. The infiltration and functions of these different cells is discussed in section 1.4.1.

1.4.1 Leukocyte subpopulations in endometrium

The number and type of leukocytes in human endometrium and decidua varies across the menstrual cycle, with implantation and throughout pregnancy. Leukocytes constitute approximately 8.2% of the stromal cells in the proliferative phase with this figure rising to 31.7% in first trimester decidua (Bulmer, Longfellow et al. 1991). Endometrial leukocytes include T and B cells, mast cells, macrophages, neutrophils

and uterine Natural Killer (uNK) cells (King, Burrows et al. 1998; Trundley and Moffett 2004). Lymphoid aggregates are present in the basalis region throughout the menstrual cycle and contain T cells and B cells surrounded by a halo of macrophages (Bulmer, Lunny et al. 1988; Bulmer 1994). T cells are also found scattered throughout the endometrial stroma and have also been localised to an intraepithelial location whilst B cells are rarely observed out with lymphoid aggregates. These cells become activated during pregnancy at the site of large lymphoid cell clusters and it is possible they are acting to provide constitutive immune defence at this critical time or control trophoblast invasion (Mincheva-Nilsson, Baranov et al. 1994). Macrophages constitute approximately 20% of the leukocytes present throughout the cycle with an increase in number observed prior to menstruation and in decidua (Kamat and Isaacson 1987; Bulmer, Lunny et al. 1988; Bonatz, Hansmann et al. 1992). A scavenger role during menstruation has been suggested. In decidua, macrophages are found at the implantation site often in contact with extravillous trophoblast (Bulmer, Lunny et al. 1988; Loke and King 1995). Macrophages are also raised in number in endometrium taken from women 48 hours after controlled progesterone withdrawal compared to decidua from pregnant women (Critchley, Jones et al. 1999). Although displaying a cyclical pattern of cell number regulation, macrophages do not express classic ERα or PR (King, Gardner et al. 1996; Stewart, Bulmer et al. 1998). Therefore, control by oestradiol and progesterone is likely to be indirect.

An influx of neutrophils occurs in the perimenstrual period at which point they comprise approximately 6-15% of total cells, and they are thought to be important in menstruation (Poropatich, Rojas et al. 1987; Salamonsen, Kovacs et al. 1999). Neutrophils produce a plethora of immunoregulatory cytokines that are reported to initiate and potentiate cellular and humoral immune responses. One example is the anti-proteinase and antimicrobial molecule elafin, which is expressed by endometrial neutrophils in a menstruation-dependent manner (King, Critchley et al. 2003). An important chemotaxis stimulus for neutrophils is IL-8 (neutrophil chemotactic factor) (Rampart, Van Damme et al. 1989; Colditz 1990). Eosinophils also rise immediately prior to menstruation and make up around 3-5% of endometrial cells at this point (Jeziorska, Salamonsen et al. 1995; Salamonsen and Lathbury 2000).

A population of phenotypically unique lymphocytes ($CD56^{bright}$, $CD16^{dim}$, $CD3^{-}$) has been reported to cluster proximal to glands and spiral arteries, with cell number altering in a cyclical manner increasing in the late secretory phase (King, Burrows et al. 1998; King 2000). These cells are uterine Natural Killer cells (uNK) and exhibit characteristic large granular lymphocyte morphology (Loke and King 1995). uNK cells are the major leukocyte population present in the endometrial stroma at the time when implantation, placentation and decidualisation occur (King 2000; Henderson, Saunders et al. 2003). Figure 1.5 depicts the leukocyte populations present in endometrium throughout the menstrual cycle.

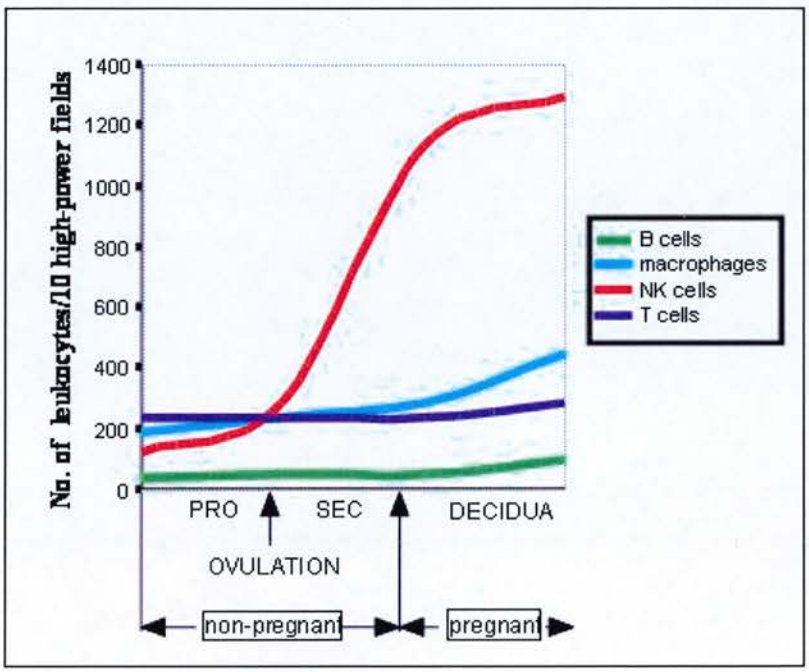


Figure 1.5: Leukocyte populations in human endometrium and first trimester decidua. Data and illustrations adapted from Loke and King, 1997.

1.4.2 Uterine Natural Killer cells

Natural killer (NK) cells constitute a major component of the innate immune system (Bancroft 1993; Scharton-Kersten and Sher 1997; Biron, Nguyen et al. 1999; Cooper, Fehniger et al. 2001). Uterine NK cell have a unique phenotype ($CD56^{bright}$, $CD16^{dim}$, CD3-), which distinguishes them from the majority of peripheral blood NK (PBNK) cells ($CD56^{dim}$, $CD16^{bright}$, $CD3^-$). CD16 is a low-affinity receptor for IgG complexes (FcRIII) and is responsible for NK-mediated antibody-dependent cellular cytotoxicity. CD56 is an isoform of the neural cell adhesion molecule (NCAM) (Nagler, Lanier et al. 1989). In addition, the CD57 antigen is also differentially expressed among NK cell subsets, with expression observed on PBNK cells but absent from uNK cells (Nagler, Lanier et al. 1989). NK cells are present in peripheral blood, accounting for approximately 10% of total blood lymphocytes (Robertson and Ritz 1990). However, the $CD56^{Bright}$ subset comprises less than 2% of total blood lymphocytes, approximately 10% of total blood NK cells (Lanier, Le et al. 1986). The exact functions of these uNK cells in humans have yet to be elucidated, but may involve a role in implantation and placentation (Loke and King 1995; Loke and King 1997; King, Burrows et al. 1998; King 2000; Loke and King 2000; Loke and King 2000).

1.4.2.1 Uterine Expression and Functions of uNK Cells

Uterine NK cells were originally termed Large Granular Leukocytes (LGLs) due to their granular lymphocyte morphology (King, Balendran et al. 1991; Loke and King 1995). Upon activation $CD56^{Bright}$ NK cells produce an array of cytokines such as IFNγ and GM-CSF, (Cooper, Fehniger et al. 2001) and demonstrate cytotoxic activity upon IL-12 or IL-2 activation (Ellis and Fisher 1989; Gately, Desai et al. 1991; Robertson, Soiffer et al. 1992). It has been demonstrated that the $CD56^{Bright}$ NK variety produce far greater levels of cytokines compared with the cytolytic $CD56^{Dim}$ cells (Cooper, Fehniger et al. 2001). However, a study comparing cytokine production by peripheral NK cells and decidual NK cells reported a very similar cytokine repertoire (Saito, Nishikawa et al. 1993). Through their secretion of cytokines it is likely uNK cells are having immunoregulatory effects. It has been

suggested that $CD56^{bright}$ uNK cells are derived from the small $CD56^{bright}$ population in peripheral blood (Dosiou and Giudice 2005). There are some notable phenotypic differences between these two populations, uNK are KIR+, $CD69^{+}$, $CD62L^{-}$, whereas $CD56^{bright}$ peripheral NK cells are KIR–, CD69–, and $CD62L^{+}$ (Moffett-King 2002; Dosiou and Giudice 2005). A recent microarray analysis identified significant differences in gene expression between the $CD56^{bright}$ uNK population in the decidua with the $CD56^{bright}$ and $CD56^{dim}$ peripheral NK cell populations, including selective expression of CD9, galectin-1, and glycodelin, in the decidual NK population but not in peripheral blood NK cells (Koopman, Kopcow et al. 2003; Dosiou and Giudice 2005). $CD56^{bright}$ uNK cells were also reported to express significantly higher levels of several other genes, including tetraspanins, integrins, lectin-like receptors, and KIRs compared with peripheral $CD56^{bright}$ cells (Koopman, Kopcow et al. 2003; Dosiou and Giudice 2005).

Several changes occur in uNK cells during the normal menstrual cycle and human pregnancy. Firstly, uNK cells are present in low numbers in the proliferative phase but increase across the secretory phase, particularly around the time of implantation (Loke and King 1997), with numbers peaking in the late secretory phase where they comprise approximately 15–25% of the endometrial stromal cells (King, Wellings et al. 1989), uNK cells are localised in clusters close to endometrial glands and spiral blood vessels (King, Burrows et al. 1998; King 2000). uNK cell numbers fall prior to menstruation. It was originally thought that uNK cells were undergoing apoptosis (King 2000) however, a more recent study has failed to provide evidence of classical apoptosis in uNK cells of the late secretory endometrium (Trundley and Moffett 2004) and their fate is therefore uncertain. In pregnancy uNK cells constitute approximately 75% of the leukocytes in 1st trimester decidua and are present until the 2^{nd} trimester with numbers subsequently declining (Bulmer, Morrison et al. 1991; Loke and King 1995). uNK cell phenotype changes across the menstrual cycle and early pregnancy (Kodama, Hara et al. 1998). Expression of CD69, HLA-DR, CD11a and CD18 are highest in the proliferative phase and decrease gradually during the menstrual cycle (Kodama, Hara et al. 1998). Similarly, a decrease in expression of

CD69, HLA-DR and CD45RA is observed during pregnancy (Kodama, Hara et al. 1998). In addition, uNK cells have been found to express one or more inhibitory receptors in early pregnancy (Ponte, Cantoni et al. 1999).

The lineage, origin, and mechanism underlying the postovulatory rise of uNK cells remain to be established. Two hypotheses exist: firstly that the increase in uNK number in mid-to-late secretory endometrium is solely the result of *in situ* proliferation and secondly that the increase is due to *de novo* migration from peripheral circulation. In support of the first, proliferation of uNK cells within the endometrium has been shown by staining with the proliferation marker, Ki67 (King, Balendran et al. 1991; Kammerer, Marzusch et al. 1999). In contrast, a precursor cell type may be selectively recruited into the endometrium, where it differentiates to become the phenotypically unique uNK cell. In support of this theory, a subset of peripheral NK cells have been identified that exhibit a similar antigenic phenotype to uNK cells (Lanier, Le et al. 1986; Marzusch, Ruck et al. 1993; Perez, Sotiropoulou et al. 2003). Chemokines are chemotactic cytokines that act via G protein-coupled receptors (GPCRs) (Murphy 1996) and endometrial chemokines have been proposed to be responsible for the increase in leukocytes in the secretory phase (Kamat and Isaacson 1987). Chemokines are known to induce leukocyte migration via activation of adhesion molecules expressed on their surface. Chemokines consist of four cysteine residues and two disulphide bonds and are split into four sub-groups according to the separation of the first two cysteines by amino acids: C, CC, CXC and CXXXC (Zlotnik and Yoshie 2000). $CD56^{bright}$ NK cells have been reported to express the chemokine receptors, CXCR3, CCR5 and CCR7 at high levels (Maghazachi 2003). Recent studies have proposed that Interleukin-8 (IL-8, CXCL8), Interleukin-15 (IL-15), Interleukin-18 (IL-18), stromal cell-derived factor (SDF-1) and macrophage inflammatory protein (MIP)-1α are potent chemoattractants for leukocytes and will be further discussed in this section.

Although the specific mechanism of uNK regulation in the uterus remains unknown, the variation in uNK cell number across the menstrual cycle suggests ovarian steroid

regulation (King, Burrows et al. 1998; Dunn, Kelly et al. 2003; Dosiou and Giudice 2005). uNK cell expansion across the progesterone-dominated secretory phase and the association of uNK cell demise with falling levels of progesterone implicates progesterone as a central regulator of their growth and this is compounded by evidence that ovariectomised women lack uNK cells (Loke and King 1995; Flynn, Byrne et al. 2000). However, a search for evidence of a direct hormonal effect on uNK cells, failed to identify either PR or ERα in human uNK cells (King, Gardner et al. 1996; Stewart, Bulmer et al. 1998; Henderson, Saunders et al. 2003). A more recent study identified expression of ERβ (full length and variant isoforms) and the glucocorticoid receptor (GR) mRNAs in purified uNK cells, but confirmed that they were negative for ERα and PR (Henderson, Saunders et al. 2003). In addition, protein expression of ERβ and the GR was confirmed (Henderson, Saunders et al. 2003). However, ER expression by uNK precursor cells would not appear to be an essential pre-requisite for successful proliferation and differentiation, at least in the mouse model, as bone marrow from ERα knockout (αERKO) and ERβ knockout (ßERKO) mice transplanted into RAG-$2^{-/-}/\gamma c^{-/-}$ mice, lacking all lymphocyte lineages, will both successfully reconstitute the uNK population (Borzychowski, Chantakru et al. 2003). The reconstituted uNK cells are reported to be capable of initiating appropriate modification of spiral arteries in the uterus (Borzychowski, Chantakru et al. 2003), but functionality has yet to be fully assessed. Therefore, a possible role for either ER in regulating uNK cell function has not yet been conclusively established.

This absence of the genomic PR suggests uNK cells are not controlled directly by progesterone but that it is possible that regulation is via paracrine signalling with non-leukocyte ESCs since these cells do express the PR A isoform during the secretory phase (Wang, Critchley et al. 1998). This is in agreement with studies where the NK cells were co-cultured with endometrial stromal cells whose treatment with progesterone upregulated the population of $CD56^{Bright}$ NK cells, whereas culture with prolactin, oestradiol or human chorionic gonadotrophin (hCG) did not induce $CD56^{Bright}$ cell proliferation (Inoue, Kanzaki et al. 1996). Interestingly, culture of highly purified endometrial $CD56^{bright}$, $CD16^{dim}$ NK cells with 17β-oestradiol or progesterone has no effect on uNK proliferation, cytotoxicity or IFNγ production

(Kitaya, Yasuda et al. 2003), in agreement with the suggestion that progesterone regulates uNK cell function in a paracrine manner via action on intermediary cells, for example, ESC or other immune cells. It is also possible that progesterone will stimulate uNK cells via the GR, which have cross-reactivity with progesterone (Kontula, Paavonen et al. 1983; Thomas, Liu et al. 2006).

1.4.2.2 Production of cytokines by uNK cells

Studies *in vitro* have demonstrated that uNK cells proliferate upon treatment with IL-15 but other, as of yet, unidentified products of ESCs appear to increase their proliferation further (Verma, Hiby et al. 2000). A study on isolated human decidual NK cells revealed these cells express mRNA and secrete the protein for G-CSF, GM-CSF, M-CSF and LIF (Saito, Nishikawa et al. 1993). This suggests that, in addition to, or in place of an immune function, uNK cells may perform other functions. For example, a close association exists between uNK cells and trophoblast cells *in vivo* providing circumstantial evidence for a role in trophoblast invasion (Loke and King 1997). In culture experiments, MIP-1α secreted from isolated human cytotrophoblast cells has been shown to attract $CD56^{Bright}$ cells (Drake, Gunn et al. 2001). The authors interpret this as MIP-1α having uNK-specific chemoattractant properties (Drake, Gunn et al. 2001). This is in contrast to studies in the murine uterus where mice genetically-ablated for MIP-1α show no difference to wild type mice with regard to uNK cell density at the implantation site (Chantakru, Kuziel et al. 2001). In addition, murine uNK cells, which are present only in the metrial gland, the triangle at the apex of the placenta where blood vessels feeding the placenta are in close proximity (Croy and Kiso 1993), express mRNA for an array of cytokines including CSF-1, TNFα, IL-1, LIF and TGFβ (Croy, Guilbert et al. 1991).

1.4.2.3 Control of uNK activity

NK cell activity is controlled by the integration of both activating and inhibitory signals. Notably, NK cells express receptors that upon engagement of class I MHC proteins deliver inhibitory signals to the NK cytolytic machinery. The most studied NK receptors are the KIRs, the immunoglobulin-like transcripts (ILTs), and the CD94/NKG2 proteins. In contrast to blood where only approximately 50% of NK cells express CD94/NKG2A, all decidual NK cells are characterised by the expression of high levels of CD94/NKG2A (Borrego, Kabat et al. 2002; Trundley and Moffett 2004). KIRs recognise groups of HLA alleles that have certain amino acids in common, and in concert with CD94/NKG2A it is thought that these receptors provide a means by which decidual NK cells differentially respond to trophoblast cells, which display a specific complement of HLAs, and maternal somatic cells expressing all the HLA class I proteins (Trundley and Moffett 2004). uNK cells display no cytolytic activity against the invading cytotrophoblast but do against the NK-sensitive cell line, K562 (King, Birkby et al. 1989).

Further evidence supporting the role of uNK cells in implantation and in the pathogenesis of early pregnancy loss is gained from studies examining women suffering from recurrent miscarriage who are reported to display both an increase in PBNK cell number (Ntrivalas, Kwak-Kim et al. 2001) and a greater infiltrate and activation of $CD56^{bright}$, $CD16^{dim}$ uNK cells *in utero* (Clifford, Flanagan et al. 1999; Quenby, Bates et al. 1999). In contrast, a study by Lachapelle *et al* demonstrated that the $CD56^{bright}$, $CD16^{dim}$ NK cell subset was significantly decreased in favour of the $CD56^{dim}$, $CD16^{bright}$ NK cell subset in all patients with recurrent spontaneous abortion (Lachapelle, Miron et al. 1996). However, a recent report evaluating the immunophenotypes of endometrial leukocytes in women with history of recurrent spontaneous miscarriage has concluded that endometrial biopsies from the peri-implantation period in a menstrual cycle prior to pregnancy cannot predict subsequent pregnancy outcome (Michimata, Ogasawara et al. 2002). These studies implicate uNK cell involvement in early pregnancy maintenance, with aberrant

expression leading to a failed pregnancy. However, these findings could alternatively represent an attempt by uNK cells to "rescue" a failing pregnancy, although no evidence is available to confirm or deny this.

1.4.2.4 uNK Cells and Decidualisation

uNK cells have been implicated in the process of decidualisation. uNK cells have been specifically localised within regions of stroma exhibiting pseudodecidual alterations (Bulmer, Lunny et al. 1988) and in the murine uterus, uNK cells are proposed to be essential for maintenance of the decidual reaction rather than its initiation (Croy, Ashkar et al. 1997; Croy, Chantakru et al. 2002; Croy, Esadeg et al. 2003). Evidence from studies on mice lacking IFNγ implicates this cytokine, a major product of uNK cells, in the decidual reaction and conversion of the uterine vasculature (Ashkar and Croy 1999; Ashkar, Di Santo et al. 2000; Ashkar and Croy 2001; Croy, Chantakru et al. 2002; Ashkar, Black et al. 2003; Croy, Esadeg et al. 2003). In contrast, *in vitro* decidualisation of cultured human ESCs is antagonised by treatment with IFNγ, implying that the role of IFNγ may differ between mice and humans (Christian, Marangos et al. 2001). As previously mentioned, prolactin is secreted by human decidualised stromal cells and regulation of prolactin receptor mRNA expression is mediated by progesterone (Tseng and Mazella 1999). A recent study has identified the prolactin receptor on uNK cells, suggesting that uNK cells are a novel target for prolactin action representing a possible functional link between uNK cells and ESCs, and providing an indirect connection between progesterone and control of uNK cells (Gubbay, Critchley et al. 2002).

1.4.3 Macrophage Mannose Receptor

The macrophage mannose receptor (MMR) was initially recognised as a receptor involved in the clearance of endogenous glycoproteins (Stahl, Rodman et al. 1978; Pontow, Kery et al. 1992). Named owing to its affinity for sugars terminated in mannose, fructose or N-acetyl glucosamine, the macrophage mannose receptor has been implicated in the recognition of exposed mannose residues on the surface of selected pathogens and the internalisation of mannosylated antigens. Therefore, these receptors may play a role in microbial recognition and antigen presentation and in lymphocyte adhesion (Wileman, Boshans et al. 1985; Taylor, Boland et al. 1991). Natural ligands for the receptor include microbial polysaccharides, and glycoproteins, such as LH and thyroid stimulating hormone (TSH), produced by the anterior pituitary, glycolipids, and mammalian glycoproteins with N-linked high-mannose (Fiete, Srivastava et al. 1991; Fiete, Beranek et al. 1997; Linehan, Martinez-Pomares et al. 2000).

The MMR (CD206), a 180 kDa transmembrane protein, was the first member of a family of four mammalian endocytic receptors to be discovered (Ancian, Lambeau et al. 1995). All members share the same structure: an extracellular N-terminal cysteine-rich (CR) domain, followed by a domain containing fibronectin type two repeats (FNII), multiple C-type lectin-like carbohydrate recognition domains (CTLDs), and a transmembrane domain and short cytoplasmic tail (Taylor, Conary et al. 1990). The MMR CTLD mediates binding to carbohydrates terminating in mannose, fructose, or N-acetylglucosamine, and the MMR CR domain recognises sugars terminating in 4-sulfated *N*-acetylgalactosamine ß1-4 linked to *N*-acetylglucosamine (SO4-4/3-GalNAc) (Fiete, Beranek et al. 1997; Fiete, Beranek et al. 1998; Leteux, Chai et al. 2000; Liu, Chirino et al. 2000). The structure of the MMR is depicted in Figure 1.6. The majority of cellular MMR is located within the endocytic pathway with only ~ 15% localised to the cell surface (Pontow, Kery et al. 1992). A soluble form, sMMR, which maintains its mannose-binding properties has been identified, and is reported to be produced by proteolytic cleavage of the cell-associated MMR form by a MMP

(Martinez-Pomares, Mahoney et al. 1998; Jordens, Thompson et al. 1999). The MMR is expressed by most tissue macrophages, but is not macrophage-restricted and has been identified in liver sinusoidal cells, non-vascular endothelia, skin dermis, mucosal lamina propria and the T-cell areas of tonsil (Shepherd, Tarnowski et al. 1991; Linehan, Martinez-Pomares et al. 1999; Wilt, Greaton et al. 1999; Engering, Geijtenbeek et al. 2002; Taylor, Gordon et al. 2005). As a consequence of its binding properties and tissue distribution, the MMR can be placed at the interface between homeostasis and immunity. Regulation of MMR expression and functionality by cytokines, immunoglobulin receptors, and pathogens has been demonstrated. For example, IFNγ is reported to modulate the functional properties of MMR and can act in synergy with IL-4 to enhance MMR-dependent uptake (Marodi, Schreiber et al. 1993; Stahl and Ezekowitz 1998). Furthermore, in recruited inflammatory peritoneal macrophages, MMR levels are increased in response to IL-4, IL-10, and IL-13 (Stein, Keshav et al. 1992; Doyle, Herbein et al. 1994; Martinez-Pomares, Reid et al. 2003). In contrast, IFNγ is reported to decrease cell surface expression of MMR and MMR-mediated endocytosis (Harris, Super et al. 1992; Raveh, Kruskal et al. 1998). However, both IL-4 and IFNγ either alone, or together, are reported to increase MMR-mediated phagocytosis (Raveh, Kruskal et al. 1998). These findings imply that The type-1 and The type-2 cytokines at sites of inflammation are able to enhance MMR-mediated phagocytosis, which may be important in the regulation of menstruation or implantation in the human uterine environment. MMR knockout mice ($MMR^{-/-}$) have a lethal phenotype and die *in utero*. Heterozygous female mice have a smaller litter size that is reported to be a result of a reduction in the rate of implantation (Mi, Shapiro et al. 2002), further supporting a role for MMR in uterine functions. In addition, when MMR binds to microorganisms, a plethora of intracellular responses have been reported, including cytokine secretion and modulation of other cell surface receptors (Shibata, Metzger et al. 1997; Yamamoto, Klein et al. 1997; Apostolopoulos, Barnes et al. 2000). Furthermore, MMR has recently been demonstrated to be involved in antigen uptake and delivery to MHC class 1 molecules (Apostolopoulos, Barnes et al. 2000; Apostolopoulos and McKenzie 2001).

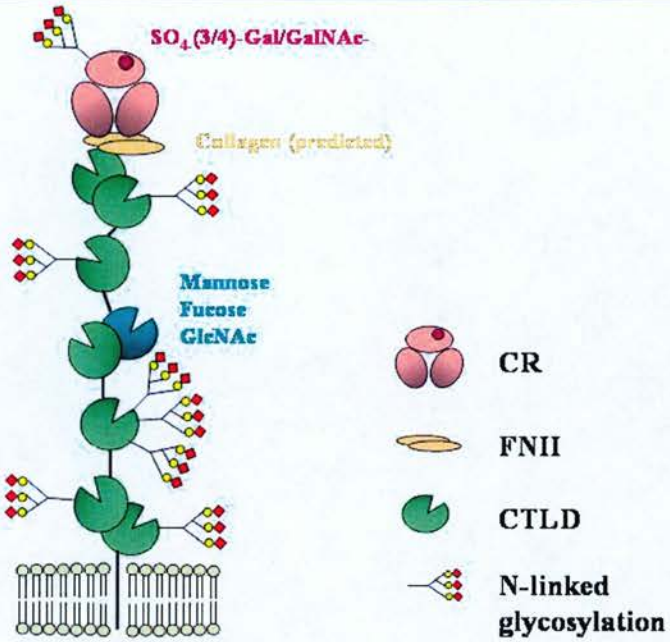


Figure 1.6: Structure of the macrophage mannose receptor. Domain structure of the MMR showing the proposed extended conformation and predicted N-linked glycosylation (proposed sialylation indicated by red diamonds). The CR domain (red), fibronectin type II (FNII) repeat (orange) and CTLD (green) are shown. CTLD4, the CTLD mostly responsible for sugar binding, is shown in dark green. Ligands for each of the binding regions have also been included (colours correspond to the binding domain where known). Redrawn from Taylor *et al* (Taylor, Gordon et al. 2005).

1.5 Cytokines

Cytokines are small glycoproteins with autocrine and paracrine interactions and are predominantly associated with immune functions. Cytokines can be involved in both the induction and resolution of an inflammatory response and are generally denoted as "pro-inflammatory" or "anti-inflammatory". In the human endometrium, during the peri-implantation phase, a reduction or absence of Th-1 and a dominance of Th-2 cytokines is observed (Lim, Odukoya et al. 1998). The general hypothesis, with regard to pregnancy, is that a Th-1 response is considered detrimental and a Th-2 response is thought to be beneficial and supportive of pregnancy by contributing to the local modulation of maternal adaptive immune response to facilitate successful embryonic implantation and maintenance of pregnancy (Wegmann, Lin et al. 1993; Hill, Polgar et al. 1995; Lim, Odukoya et al. 1998; Hill and Choi 2000). Some of the

reproductive functions of cytokines are summarised in Table 1.1. The cytokines covered in this section are reported to be involved in decidualisation and/or leukocyte recruitment.

Cytokine	Endometrial function
IL-1	Induction of adhesion molecules Modulation of proliferation Alteration of morphology Induction of cytokines Chemotaxis and induction of lymphoid infiltration Oedema Induction of PGE_2 Activation of T cells
TGFβ1	Gland formation and Angiogenesis Extracellular matrix formation Modulating myofibroblast differentiation Tissue remodelling and homeostasis Regulation of TIMPs
IFNγ	Induction of adhesion molecules Modulation of proliferation Alteration of morphology Induction of cytokines Chemotaxis and induction of lymphoid infiltration Induction of HLA-DR and ICAM-1
TNFα	Induction of adhesion molecules Modulation of proliferation Alteration of morphology Induction of cytokines Chemotaxis and induction of lymphoid infiltration Oedema Induction of PGE_2 Injury to endometrial vessels Activation of polymorphonuclear leukocytes

Table 1.1: Cytokine functions in the human endometrium. Adapted from Tabibzadeh 1994 (Tabibzadeh 1994).

1.5.1 Cytokines involved in decidualisation and/or leukocyte recruitment and proliferation

1.5.1.1 Activin

Activins are dimeric proteins consisting of two subunits (ßA and ßB) and they can exist as ßA - ßA or ßB - ßB homodimers or the ßA - ßB heterodimer (Ying 1987; Schwall, Nikolics et al. 1988). Activins can be inactivated by binding to follistatin (Vale, Rivier et al. 1986; Ying 1987; Shimonaka, Inouye et al. 1991; Schneyer, Rzucidlo et al. 1994) and act via the ActRIIB and ActRII receptors found on plasma membranes (Massague 1992; Mathews and Vale 1993). Activins are highly expressed in the endometrium, with both activin subunits primarily localised to endometrial glands (Leung, Salamonsen et al. 1998; Otani, Minami et al. 1998; Petraglia, Florio et al. 1998; Jones, Brauman et al. 2000), and maximal levels seen in the secretory phase. Activin ßA and ßB subunits are both dramatically upregulated in both *in vivo* and *in vitro* models of decidualisation (Otani, Minami et al. 1998; Jones and Critchley 2000) and are highly expressed in the extensively decidualised endometrium induced by intrauterine delivery of progestin (Jones and Critchley 2000; Roopa, Loganath et al. 2003). Furthermore, activin A promotes decidualisation *in vitro*, while neutralisation of activin action by treatment with follistatin significantly inhibits the decidual response (Jones, Salamonsen et al. 2002; Tierney, Tulac et al. 2003). Activin ßA subunits are intensely expressed by neutrophils and macrophages in the premenstrual and menstrual endometrium and have been proposed to have roles in menstruation and tissue regeneration (Leung, Salamonsen et al. 1998).

1.5.1.2 Interleukin-1 (IL-1)

IL-1 is a pleiotropic cytokine which exists in two isoforms: IL-1α and IL-1β, they have similar action and act via the same receptor, IL-1R type-1. A second receptor sub-type, known as IL-1R type-2, exists and is found on B cells, neutrophils and monocytes. This receptor preferentially binds IL-1β (Colotta, Re et al. 1993) and may act as a decoy inhibitor of both isoforms of IL-1 (Colotta, Re et al. 1993). In the

human endometrium IL-1β mRNA levels are undetectable in proliferative and early secretory phase endometrium, whereas both IL-1α and IL-1β are expressed in the epithelial and stromal cells of secretory phase endometrium (Tabibzadeh and Sun 1992) and 1st trimester decidua (Kauma, Matt et al. 1990). Human serum IL-1 levels are cyclically regulated and are reported to be highest in the secretory phase, post-ovulation (Cannon and Dinarello 1985). IL-1 has also been implicated in blastocyst implantation and is expressed in conjunction with the receptor in both trophoblast and decidual cells (Simon, Frances et al. 1994; Simon, Frances et al. 1994; Simon, Frances et al. 1995; Simon, Pellicer et al. 1995). ESCs have been reported to generate an oestrogen receptor-dependent immune-related response upon treatment with IL-1α (Ruiz, Montes et al. 1997; Pioli, Weaver et al. 2006), which was reduced when cells were cultured under decidualising conditions (Ruiz, Montes et al. 1997).

It has also been suggested that IL-1 may also be involved in the initiation of menstruation by stimulating production of MMPs and PGs (Cole, Seki et al. 1995; Ishihara, Matsuoka et al. 1995; Singer, Marbaix et al. 1997; Kawano, Nakamura et al. 2001; Nishiura, Noda et al. 2005; Rossi, Sharkey et al. 2005). In addition, aberrant production of IL-1 has been implicated in disorders or menstruation and pregnancy, such as impaired decidualisation (Mizuno, Tanaka et al. 1999), pre-eclampsia (Lockwood, Matta et al. 2006) and endometriosis (Fakih, Baggett et al. 1987; Mori, Sawairi et al. 1992; Lebovic, Bentzien et al. 2000; Hudelist, Lass et al. 2005; Yin, Sun et al. 2006).

1.5.1.3 Interleukin-8 (IL-8)

IL-8 has been implicated in angiogenesis, mitogenesis of epidermal cells and chemotaxis of leukocytes (Koch, Polverini et al. 1992; Tuschil, Lam et al. 1992). In the human uterus, IL-8 has been localised to the perivascular cells in the late secretory phase and first trimester decidua (Critchley, Kelly et al. 1994; Critchley, Kelly et al. 1996; Critchley, Jones et al. 1999; Milne, Critchley et al. 1999), choriodecidual cells (Kelly, Leask et al. 1992) and in amniotic fluid (Laham, Rice et al. 1993). *In vitro* culture of human ESCs and epithelial cells has demonstrated that

IL-1α and TNFα regulate IL-8 production (Arici, Head et al. 1993). Progesterone has been proposed to be a primary regulator of IL-8 expression and in human endometrial explant studies, progesterone suppressed IL-8 secretion (Kelly, Illingworth et al. 1994). This is in agreement with *in vivo* data demonstrating an increase in IL-8 immunostaining in human endometrium 48 hours after progesterone withdrawal in a study designed to stimulate the hormonal milieu of the late secretory phase (luteal regression) (Critchley, Jones et al. 1999). Together, these studies provide evidence to implicate IL-8 in regulating menstruation (Kelly, Illingworth et al. 1994; Critchley, Jones et al. 1999).

1.5.1.4 Interleukin-10 (IL-10)

IL-10 is a pleiotropic homodimeric cytokine secreted by leukocytes and somatic cells with well-characterised anti-inflammatory and immune-deviating properties (Chabot, Williams et al. 1999; Moore, de Waal Malefyt et al. 2001). IL-10 favours differentiation and function of lymphocyte subsets with pivotal roles in immune tolerance (Enk, Saloga et al. 1994). Moreover, IL-10 terminates inflammatory responses and limits inflammation-induced tissue pathology by inhibiting synthesis of TNFα, IL-1, and a large array of other pro-inflammatory cytokines and chemokines in monocytes/macrophages (Moore, de Waal Malefyt et al. 2001). IL-2-induced $CD56^{Bright}$ NK cells are reported to proliferate and produce cytokines upon treatment with IL-10 (Carson, Lindemann et al. 1995). The specific role of IL-10 in reproduction is unclear, however IL-10 is among the cytokines aberrantly expressed in decidual T-lymphocyte populations of women with unexplained recurrent miscarriage (Piccinni, Beloni et al. 1998) and reduced IL-10 immunostaining was observed in uNK cells from women with spontaneous abortion compared with those from elective terminations (Plevyak, Hanna et al. 2002). In contrast to this, IL-10 production was increased in stimulated peripheral blood mononuclear cells from women with recurrent pregnancy loss compared with those from pregnant and non-pregnant women (Bates, Quenby et al. 2002).

1.5.1.5 Interleukin-11 (IL-11)

IL-11 is a member of the gp130 cytokine family (Gadient and Patterson 1999) and is reported to have effects in a wide range of environments including, haematopoietic cells, the nervous system, and the gastrointestinal tract (Hawley 1994; Sands, Bank et al. 1999). Within the human endometrium, IL-11 is expressed in all cell types with greatest immunostaining intensities apparent in decidualised stroma, appearing before prolactin (Dimitriadis, Salamonsen et al. 2000). In culture, human ESCs produce IL-11 and express IL-11 receptor α (IL-11Rα) during progesterone-induced decidualisation (Dimitriadis, Robb et al. 2002). Moreover, treatment with an anti-human IL-11 antibody results in a reduction in production of prolactin and IGFBP-1 implicating IL-11 in the induction and regulation of decidualisation (Dimitriadis, Robb et al. 2002). In the murine uterus, IL-11 appears to be essential for female fertility. Mice knocked out for the IL-11Rα gene have a defective uterine response to the blastocyst which fails to trigger a normal decidual transformation (Bilinski, Roopenian et al. 1998; Robb, Li et al. 1998).

1.5.1.6 Interleukin-15 (IL-15)

IL-15, a member of the 4 α-helix cytokine family, exists in two forms due to alternative splicing (Fehniger and Caligiuri 2001). The receptor complex for IL-15 is composed of three subunits. The α-chain is unique to its receptor but both the -β and γ-chains are common to the IL-2 receptor (Carson, Giri et al. 1994; Grabstein, Eisenman et al. 1994). Both the message and protein levels of IL-15 are increased by IFNγ and by ligation with CD-40 (Weiler, Rogashev et al. 1998; Weiler, Kachko et al. 2001). Although the actions of IL-2 and IL-15 are reported to be similar, their tissue distribution is distinct. The uterus and placenta were found to be negative for IL-2 (Jokhi, King et al. 1994; King, Jokhi et al. 1995), but positive for IL-15 mRNA and protein (Kitaya, Yasuda et al. 2000; Okada, Okada et al. 2000; Verma, Hiby et al. 2000). Gene profiling has demonstrated a three fold up-regulation of the IL-15 precursor, IL-15 and the α chain of the IL-15 receptor during the human "implantation window" compared with the late proliferative phase, supporting the

role of IL-15 in the role of implantation (Kao, Tulac et al. 2002; Giudice 2004). This pattern of expression has been confirmed by other studies examining IL-15 mRNA and protein expression across the cycle (Kitaya, Yasuda et al. 2000; Okada, Okada et al. 2000; Verma, Hiby et al. 2000; Chaouat, Zourbas et al. 2002; Dunn, Critchley et al. 2002). Expression of IL-15 is not limited to the cycling endometrium but has also been observed in abundance in 1st trimester decidua (Kitaya, Yasuda et al. 2000; Okada, Okada et al. 2000). In *in vitro* studies of human endometrial stromal cells, incubation with either progesterone or a combination of progesterone and oestradiol has resulted in enhancement of IL-15 production (Okada, Nakajima et al. 2000). Furthermore, human decidual NK cells express the IL-15 receptor, in response to IL-15 proliferate, and augment their cytolytic activity against an NK-sensitive cell line, K562 (Verma, Hiby et al. 2000). uNK development and transformation of uterine vasculature has been reported to be defective in IL-15 knockout mice (-/-) (Greenwood, Minhas et al. 2000; Kennedy, Glaccum et al. 2000; Miyazaki, Tanebe et al. 2002), further supporting the concept that uNK cells are involved in transformation of uterine vasculature (King and Loke 1990). Taken together, these studies support a role for IL-15 as the cytokine responsible for uNK cell proliferation *in vivo*; it may therefore contribute to the increase in uNK cell numbers in the secretory and early pregnancy endometrium by stimulating uNK proliferation.

1.5.1.7 Interleukin-18 (IL-18)

IL-18 has been reported to be implicated in the uNK activation pathway during the implantation process. In mice, IL-18 was detected in decidual cells immediately after implantation. However, after gestation day (GD) 8, IL-18 was produced solely by uNK cells, and it has been suggested that uNK cells are initially activated by the stromal compartment and thereafter, lymphocyte-derived signals maintain uNK cell activation (Croy, Esadeg et al. 2003; Zhang, He et al. 2003). In a study using secretory phase biopsies either from women who failed to become pregnant after repeated IVF transfer, or from control fertile patients, the authors report the number of $CD56^{Bright}$ cells were significantly different in fertile and infertile patients and these differences were significantly correlated with IL-15 and IL-18 mRNA levels,

further intimating that both IL-15 and IL-18 are intimately involved in the regulation of uNK cells *in vivo* (Ledee-Bataille, Bonnet-Chea et al. 2005). Furthermore, IL-18 has been demonstrated to be negatively regulated by exogenous ovarian steroid hormone treatment in women (Ledee, Dubanchet et al. 2006). Interestingly, IL-18 mRNA expression was not reported to change over two natural cycles, as measured on day 21 of the cycle (Ledee, Dubanchet et al. 2006). In addition, Th-1 associated cytokines, IFNγ, IL-12 and IL-18, are increased in 1st trimester decidua in women during miscarriage (Wilson, Moor et al. 2004).

1.5.1.8 Interferon γ (IFNγ)

IFNγ is expressed by many cell types, including T cells (Sugawara, Kitagawa et al. 1986) and NK cells (Peritt, Robertson et al. 1998; Loza, Zamai et al. 2002; Perussia, Chen et al. 2005). IFNγ produced by murine NK cells has been suggested as a mediator for promoting the development of Th-1 T cell responses and activation of pro-inflammatory macrophages (Martin-Fontecha, Thomsen et al. 2004). The source of IFNγ within the human endometrium is thought to be the lymphoid aggregates (Tabibzadeh 1994), polymorphonuclear neutrophils in non-pregnant endometrium (Yeaman, Collins et al. 1998), and uNK cells in first trimester decidua (Saito, Nishikawa et al. 1993; Jokhi, King et al. 1994). IFNγ levels remain consistent across the menstrual cycle in human non-pregnant endometrium (Yeaman, Collins et al. 1998). In the pregnant mouse IFNγ can only be detected in supernatants from first trimester decidua after which it fails to be detected (Lin, Mosmann et al. 1993). IFNγ is reported to induce an increase in the levels of IL-6, MCP-1 and MCSF and reduce IL-8 levels in cultures of ESCs, suggesting that IFNγ produced by both decidual inflammatory cells and the developing embryo could play a role in the maintenance of early pregnancy by modulating the production of these cytokines by ESCs *in vivo* (Nasu, Matsui et al. 1998). In a recent study using an ERα null mouse, oestradiol was shown to selectively enhance the development of IFNγ-producing T-cells, through an ERα-dependent mechanism (Maret, Coudert et al. 2003). Moreover, oestrogen is reported to increase activity of the IFNγ promoter and has been demonstrated to increase expression of IFNγ mRNA in ConA-treated murine spleen cells (Fox, Bond

et al. 1991). Further experiments with IFNγ null mice have identified that IFNγ is a primary mediator of murine uNK cell function (Ashkar and Croy 2001). In addition to its effects on the placental vasculature in the mouse, IFNγ induces human trophoblast cells to become partially protected from lysis by IL-2 stimulated decidual NK cells (King and Loke 1993).

1.5.1.9 Monocyte Chemotatic Protein-1 (MCP-1)

Monocyte chemotactic protein-1 (MCP-1) (CCL2) is a product of a plethora of cell types including fibroblasts (Yoshimura and Leonard 1990), endothelial cells (Sica, Wang et al. 1990), and monocytes (Yoshimura, Robinson et al. 1989). MCP-1, as its name implies, is a chemotactic agent for monocytes (Rollins and Pober 1991). MCP-1 is co-expressed with IL-8 and COX-2 in perivascular cells in the human endometrium in the late secretory phase (Jones, Kelly et al. 1997), suggesting a role for these molecules in regulating perimenstrual infiltration of leukocytes. Cultures of human ESCs and epithelial cells secrete MCP-1 (Arici, MacDonald et al. 1995) and have been shown to respond to IFNγ and CD40 ligand by releasing MCP-1 after 24 hours of treatment (King, Kelly et al. 2001). However, oestrogen has been shown to inhibit secretion of MCP-1 by ESCs (Arici, MacDonald et al. 1995; Arici, Senturk et al. 1999) and production by choriodecidual cells and the breast cancer cell line, T47D, is suppressed by progesterone (Kelly, Carr et al. 1997). Steroid hormone regulation was also reported by Arici *et al* who demonstrated that progesterone treatment inhibited MCP-1 production in ESC, but this did not seem to be as potent as oestradiol treatment (Arici, Senturk et al. 1999). However, a more recent study has failed to replicate the inhibitory effects of progesterone on MCP-1 mRNA levels in cultured human ESCs or epithelial cells (Arici, Senturk et al. 1999; DeLoia, Stewart-Akers et al. 2000). MCP-1, in combination with oestradiol, has also been demonstrated to augment the protein release of VEGF from ESC in culture (Lin and Gu 2005).

1.5.1.10 Macrophage Inflammatory protein-1β

Recent studies have suggested a potential role for MIP-1β (CCL4) in the mechanism of uterine recruitment of PBNK cells (Cook 1996; Kitaya, Nakayama et al. 2003). The chemokine, MIP-1β, has strong chemoattractant properties for PBNK cells. MIP-1β expression is reported to increase in the secretory phase of the menstrual cycle, in response to increasing progesterone levels (Kitaya, Nakayama et al. 2003). Furthermore, MIP-1β secretion by cultured ESCs can be induced *in vitro* by progesterone. The increase in MIP-1β expression observed in the secretory phase parallels the increase in uNK cell number (Kitaya, Nakayama et al. 2003). The specific receptor for MIP-1β, CCR5, is strongly expressed by uNK, consistent with a role for MIP-1β as a chemoattractant for $CD56^{bright}$ NK cells, during the normal menstrual cycle and in pregnancy (Red-Horse, Drake et al. 2001; Kitaya, Nakayama et al. 2003). A recent microarray-based study has identified MIP-1β as a chemokine upregulated during endometrial receptivity and early pregnancy (Jones, Hannan et al. 2004).

1.5.1.11 Tumor necrosis factor α (TNFα)

Tumor necrosis factor α (TNFα) is an inflammatory cytokine (Old 1985) with wide ranging actions including, monocyte chemotaxis (Ming, Bersani et al. 1987 6951) and angiogenic effects (Frater-Schroder, Risau et al. 1987). Cyclic changes in TNFα have been reported in the human endometrium which has been localised to both glandular epithelium and stroma (Hunt, Chen et al. 1992) with maximal stromal expression observed in the late secretory phase (Hunt, Chen et al. 1992). Tabibzadeh *et al* (Tabibzadeh, Zupi et al. 1995) described increasing TNFα production throughout the cycle with a peak during menstruation, suggesting a role in menstruation due to the ability of the cytokine to induce apoptosis and compromise vascular integrity (Tabibzadeh, Zupi et al. 1995; Tabibzadeh 1996). Steroid hormone control of TNFα expression has been described with progesterone suppression of TNFα production reported in activated mouse macrophages (Miller and Hunt 1998)

and in human endometrial epithelial cells derived from the secretory phase of the cycle (Laird, Tuckerman et al. 1996). TNFα is reported to be produced from uNK cells (Saito, Nishikawa et al. 1993) and to induce synthesis of IL-8 (Sica, Matsushima et al. 1990; Ito, Nakamura et al. 1994; Goldstein, Strieter et al. 1996; Brasier, Jamaluddin et al. 1998). TNFα has also been reported to inhibit *in vitro* decidualisation of ESCs and prolactin release from term decidual cells (Inoue, Kanzaki et al. 1994; Jikihara and Handwerger 1994). TNFα is elevated in pathologic conditions such as endometriosis and has been shown to exhibit embryo toxicity (Taketani, Kuo et al. 1992).

1.5.1.12 Transforming Growth Factor β (TGFβ)

Transforming Growth Factor β (TGFβ) belongs to a superfamily of structurally related regulatory proteins; TGFβ1, TGFβ2, TGFβ3, TGFβ4 and TGFβ5, activins/inhibins, Müllerian inhibiting substance (MIS), bone morphogenic proteins (BMP), and products of the Xenopus Vg1 and Drosophila decapentaplegic (dpp) genes (Reviewed in (Rizzino 1988; Massague 1990; Sporn and Roberts 1990)). TGFβ1, TGFβ2 and TGFβ3 are all expressed in mammals (Lawrence 1996).

TGFβs are multifunctional cytokines responsible for a plethora of cellular processes. They are reported to regulate such diverse functions as cell proliferation, differentiation and physiological events such as angiogenesis, immune function, steriodogenesis and tissue remodelling and repair (Sporn, Roberts et al. 1987; Barnard, Lyons et al. 1990; Moses, Yang et al. 1990; Ruscetti and Palladino 1991; Wahl 1991; Sporn and Roberts 1992; Wahl 1992). Since all of these events occur in the uterine endometrium during the menstrual cycle and pregnancy, TGFβ has been implicated in regulating many reproductive functions (Godkin and Dore 1998).

The TGFβ isoforms display amino acid sequence homologies in the order of 70 – 80 % (Derynck, Jarrett et al. 1986). TGFβ1 is expressed almost ubiquitously whilst the other isoforms are expressed in a more specific manner. All the isoforms of TGFβ are encoded as large precursor proteins that are 390–412 amino acids in length (Derynck,

Jarrett et al. 1985; Gentry and Nash 1990); each isoform is the product of a separate gene (Derynck, Jarrett et al. 1985; Gentry and Nash 1990). Mature TGFβ proteins undergo a series of processing steps prior to their secretion from the cell. The most important processing step appears to be the proteolytic digestion of the precursor by the endopeptidase furin, which cleaves the TGFβ protein between amino acids 278 and 279 (Dubois, Laprise et al. 1995; Blanchette, Day et al. 1997). This proteolysis yields two products that assemble into dimers. The 65–75-kDa protein released from the N-terminal region is called the latency-associated peptide (LAP), while the second 25-kDa from the C-terminal portion of the precursor is called the mature TGFβ (Brown, Wakefield et al. 1990; Gentry and Nash 1990; Lawrence 1996; Munger, Harpel et al. 1997). Despite the cleavage of the precursor a common feature of all TGFβs is that the N-terminal portion remains noncovalently associated with the rest of the protein (Lawrence 1996; Munger, Harpel et al. 1997; Khalil 1999; Khalil 2001). The presence of the LAP protein facilitates transit of TGFβ from the cell (Lopez, Cook et al. 1992) and makes the TGFβ biologically inactive. Other post-translational modifications of TGFβ that take place before secretion include glycosylation, mannose-6-phosphorylation of the glycoprotein and disulfide isomerisation (Brunner, Gentry et al. 1988; Gentry, Lioubin et al. 1988; Purchio, Cooper et al. 1988). These are processing steps and are distinct from activation events required for the generation of biologically active TGFβ from the latent precursor.

1.5.1.12.1 Activation of TGFβ

In vitro the LAP from all isoforms of latent TGFβ (LTGFβ) can be removed by extremes of pH, such as 2 or 8, heat such as 100 °C, chaotropic agents, these are agents which may distrupt the molecular structure, and substances like SDS and urea (Brown, Wakefield et al. 1990; Lawrence 1996; Munger, Harpel et al. 1997). In addition, many physiological substances have been reported to activate LTGFβ *in vitro*. Some examples are the serine protease, plasmin, other proteases such as endoglycosidase F, sialidase, neuraminidase, cathepsins B and D, calpain, and the

glycoprotein, thrombospondin-1 (Miyazono and Heldin 1989; Oursler, Riggs et al. 1993; Schultz-Cherry, Chen et al. 1995; Khalil, Corne et al. 1996; Khalil 1999).

Plasmin is derived from plasminogen by the enzymatic action of urokinase plasminogen activator (uPA) or tissue-type plasminogen activator (tPA) (Allan and Martin 1995; Reuning, Magdolen et al. 1998). uPA activation of plasminogen to plasmin occurs while uPA is bound to a specific cell surface receptor urokinase plasminogen activator receptor (uPAR). The binding of tPA to a cell membrane receptor has not yet been confirmed, but tPA may be the major plasminogen activator involved in release of plasmin from a blood clot (Allan and Martin 1995; Reuning, Magdolen et al. 1998). tPA and uPA are not only inducible, but their activity can also be inhibited by the actions of plasminogen activator inhibitors (PAI) which exist in at least four isoforms (Vaughan 1998). Receptor-bound uPA can be inactivated by PAI-1 when the PAI-1/uPAR/uPA complex is internalised (Lyons, Keski-Oja et al. 1988; Allan and Martin 1995; Reuning, Magdolen et al. 1998; Vaughan 1998). Once cell surface expression of uPA is diminished, the generation of plasmin is also reduced (Lyons, Keski-Oja et al. 1988; Allan and Martin 1995; Khalil, Corne et al. 1996; Reuning, Magdolen et al. 1998; Vaughan 1998; Khalil 1999). A previous study reported that TGFβ1 stimulates the synthesis of PAI-1, demonstrating a negative feedback loop on its own production. In addition, progesterone has been shown to regulate the release of PAI-1 in stromal cells *in vitro* (Casslen, Andersson et al. 1986). Furthermore, uPA is expressed in the late secretory phase in co-ordinance with falling progesterone levels prior to menstruation (Casslen, Sandberg et al. 1998), possibly indicating the existence of a relationship between progesterone and TGFβ1 (Sandberg, Eriksson et al. 1997). The activation of TGFβ1 is summarised in Figure 1.7.

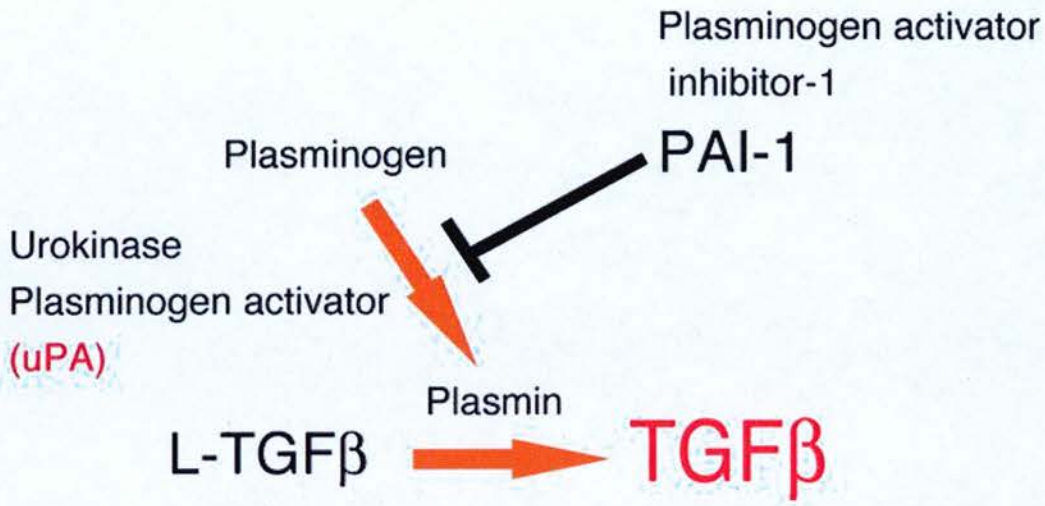


Figure 1.7: Diagrammatic representation of the proposed mechanism for the activation of TGFβ from its latent to active form. Plasminogen is cleaved by uPA to form plasmin, which in turn activates latent TGFβ (L-TGFβ) to its active form. In turn, uPA is inhibited by PAI-1.

1.5.1.12.2 TGFβ1

TGFβ1, the prototype member of the TGFβ superfamily, is a highly conserved molecule, indeed, mature human, porcine, chicken, simian and bovine TGFβ1 are identical and the amino acid sequence between human and mouse differs by only one residue (Derynck, Jarrett et al. 1986). The biologically active 25 kDa TGFβ1 molecule consists of two identical disulfide-linked 12.5 kDa polypeptide chains (Assoian, Komoriya et al. 1983). The 12.5 kDa monomer contains 112 amino acids and, as previously described, is synthesised as a larger latent TGFβ molecule of 390 amino acid residues (Derynck, Jarrett et al. 1985). The carboxy-terminal 12.5 kDa mature peptide is proteolytically cleaved from the amino-terminal at arginine 278 (Derynck, Jarrett et al. 1985; Gentry, Webb et al. 1987). TGFβ1 is reported to regulate biological functions as diverse as embryogenesis, proliferation, differentiation and extracellular matrix remodelling (Ignotz and Massague 1986), including angiogenesis (Fajardo, Prionas et al. 1996; Sankar, Mahooti-Brooks et al. 1996; Sandberg, Casslen et al. 1998) and modulating myofibroblast differentiation (Desmouliere, Geinoz et al. 1993; Jester, Barry-Lane et al. 1996; Serini, Bochaton-

Piallat et al. 1998; Lewis, Lygoe et al. 2004; Shephard, Martin et al. 2004). TGFβ1 has been shown to enhance tissue remodelling and homeostasis in endometrial cells (Bruner, Rodgers et al. 1995; Ulloa, Creemers et al. 2001) and inactivation of TGFβ1 has been implicated in endometrial carcinogenesis (Parekh, Gama et al. 2002). Previous studies have identified that endometrial tissues as well as endometrial stromal cells in culture express TGFβ type I and type II receptor mRNA and protein (Chegini, Zhao et al. 1994; Tang, Zhao et al. 1994; Dumont, O'Connor-McCourt et al. 1995; Piestrzeniewicz-Ulanska, Brys et al. 2002). It has previously been reported that the level of TGFβ1 mRNA is higher in 1st trimester decidua as compared to endometrial tissue from the proliferative and secretory phases of the menstrual cycle (Kauma, Matt et al. 1990; Selick, Horowitz et al. 1994) and TGFβ type I receptors have been identified in term placenta (Dungy, Siddiqi et al. 1991; Mitchell and O'Connor-McCourt 1991; Mitchell, Fitz-Gibbon et al. 1992; Schilling and Yeh 2000).

1.6 Signalling cascades

Although the steroid hormones exert a fundamental influence on the regulation of inflammatory mediator expression in endometrium the precise mechanisms involved remain unclear. The promoter regions for several cytokines do not contain GRE/PRE response elements suggesting that progesterone cannot act directly to suppress expression and, equally, progesterone withdrawal cannot directly influence upregulation (McKay and Cidlowski 1999). Cross-talk between the steroid hormone signal transduction pathways and other pathways is likely.

1.6.1 TGFβ1/SMAD signalling

TGFβ1 initiates its diverse cellular responses by stimulating formation of specific heteromeric complexes of type I (ALK 5) and type II serine/threonine kinase transmembrane receptors located at the cell surface (Massague 1990; Massague 1992; Massague and Wotton 2000). The type II receptor phosphorylates type I in the juxtamembrane region (GS domain) rich in glycine and serine residues, which in turn propagates the signal intracellularly via the phosphorlation of highly conserved

members of receptor-regulated SMAD (Sma- and mothers against decapentaplegic (MAD) -related protein) family of transcriptional regulators, SMAD 2 and 3 (Graff, Bansal et al. 1996; Macias-Silva, Abdollah et al. 1996; Nakao, Imamura et al. 1997; Massague and Wotton 2000; Shi and Massague 2003). Access of SMAD 2 and 3 to the type I receptors is facilitated by auxiliary proteins, e.g. SMAD anchor for receptor activation (SARA) (Shi and Massague 2003), a cytoplasmic protein that specifically interacts with non-activated SMAD 2 and the receptor complex, thereby forming a link between the receptor and SMAD 2, facilitating phosphorylation (Tsukazaki, Chiang et al. 1998). Activated receptor-regulated SMADs form hetero-oligomeric complexes with the common mediator SMAD, SMAD 4 (Zhang, Feng et al. 1996; Kawabata, Inoue et al. 1998; Correia, Chacko et al. 2001). SMAD 4 has been localised to the cytoplasm and requires oligomerisation with the receptor-regulated SMADs in order to accumulate to the nucleus (Macias-Silva, Abdollah et al. 1996; Nakao, Imamura et al. 1997), where it controls gene expression in a cell-type-specific and ligand dose-dependent manner mediated via interactions with transcription factors, coactivators and corepressors (Liu, Pouponnot et al. 1997). Inhibitory SMADS, SMAD 6 and 7, act in an opposing manner to receptor-regulated SMADs and antagonise signalling. They are reported to compete with receptor-regulated SMADs for binding to activated type I receptors (Shi and Massague 2003). They have subsequently been demonstrated to recruit E3-ubiquitin ligases, SMAD ubiquitination regulatory factor (Smurf)1 and 2, to the activated type I receptor, thus facilitating receptor ubiquitination and subsequent degradation, thereby inhibiting the signalling pathway (Shi and Massague 2003). The SMAD signalling pathway is summarised in Figure 1.8.

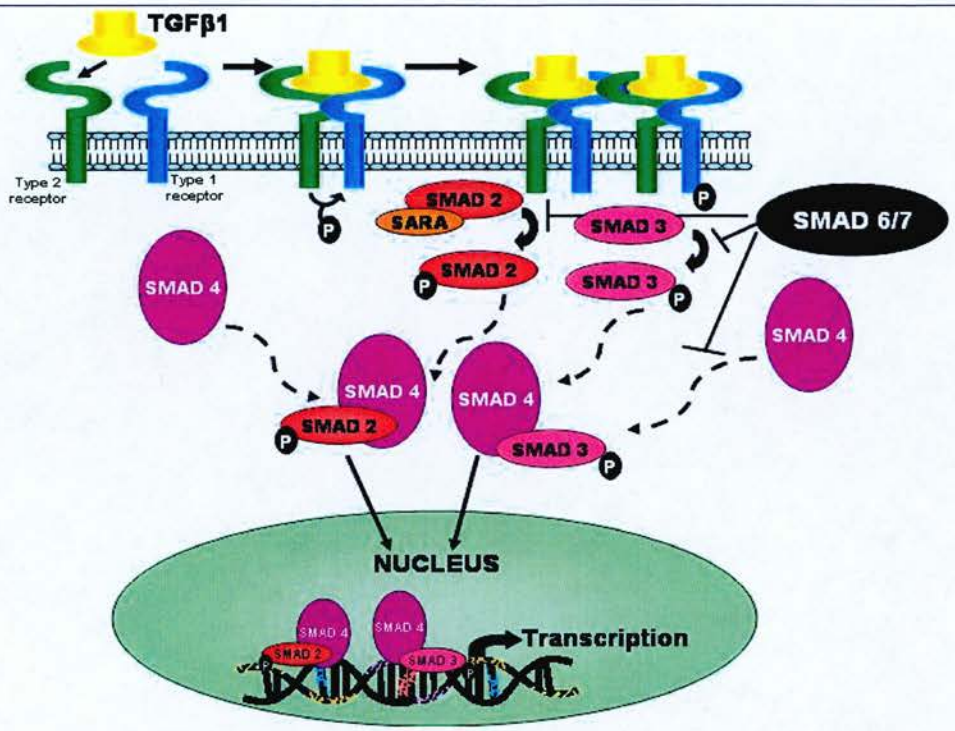


Figure 1.8: Activation and inactivation of TGFβ1-SMAD signalling. Binding of transforming growth factor β (TGFβ) to its cell surface receptor Type II leads to the phosphorylation of the Type I receptor by Type II. The Type I receptor is then able to phosphorylate and activate the receptor-regulated SMAD 2/3 (R-SMAD) protein, which is recruited to cell membrane by SARA. After activation of type I receptor, R-SMAD, is phosphorylated and forms a hetero-complex with common SMAD 4 (Co-SMAD). Subsequently, the hetero-complex translocates into the nucleus and regulates the transcription of target genes. Inhibitory SMAD 6/7 (I-SMAD) negatively regulates SMAD signalling by blocking the binding of R-SMAD to type I receptor, hetero-complex formation between R-SMAD and Co-SMAD, and the transcriptional regulation by R-SMAD in the nucleus. Self prepared.

1.6.2 Wnt- signalling pathway

Recent microarray analysis of human endometrial gene expression during the implantation window demonstrate expression and regulation of members of the Wingless/INT-1-related (Wnt) signalling pathway, implying a role for the Wnt-family in endometrial maturation and function (Carson, Lagow et al. 2002; Borthwick, Charnock-Jones et al. 2003; Kao, Germeyer et al. 2003). Wnts are a family of highly conserved lipid-modified signalling proteins (Willert, Brown et al. 2003), pivotal in influencing cell proliferation and differentiation, embryogenesis and

epithelial-mesenchymal cross-talk (Nusse and Varmus 1992; Cadigan and Nusse 1997; Wodarz and Nusse 1998). To date, at least 22 Wnts have been identified (Church, Nohno et al. 2002; Miller 2002). Signalling is initiated by Wnt ligands binding to two distinct families of cell-surface receptor molecules, Frizzed (Fzd) proteins (Bhanot, Brink et al. 1996) and low-density lipoprotein (LDL) receptor-related proteins 5 and 6 (LRP 5/6) (Rattner, Hsieh et al. 1997; Bafico, Gazit et al. 1999). The Fzd receptors, of which ten have been identified in humans to date, are a family of seven-membrane-spanning domain receptors (Bafico, Gazit et al. 1999). The LRP co-receptors are also essential for Wnt-signalling however, the mechanism as to how these receptors mediate their effects is as yet unknown. Three distinct intracellular pathways transduce Wnt signals: the canonical pathway which targets β-catenin signalling, the Wnt/Ca2+ pathway and the Wnt/polarity pathway. In the canonical pathway, downstream of Wnt1, Wnt3a, Wnt7a and Wnt8c ligand binding to Fz receptors at the cell surface activate Dishevelled (Dsh) leading to the inactivation of glycogen synthase kinase-3β (GSK-3β) by phosphorylation (Willert, Shibamoto et al. 1999). This results in the cytoplasmic accumulation of β-catenin (Miller, Hocking et al. 1999; Weidinger and Moon 2003), which forms a complex with its DNA binding partners, T cell factor (TCF) and lymphocyte enhancer factor-1 (LEF-1), and subsequent translocation to the nucleus where it regulates gene transcription (Miller, Hocking et al. 1999; van Noort and Clevers 2002; van Noort, Meeldijk et al. 2002; Weidinger and Moon 2003). In the absence of Wnt-signalling, β-catenin levels are regulated by activated GSK-3β, which phosphorylates adenomatous polyposis coli (APC) and Axin, thereby increasing their binding affinities to β-catenin, identifying it for degradation by N-terminal phosphorlyation (Nakamura, Hamada et al. 1998; Willert and Nusse 1998; Nusse 1999; Staal, Noort Mv et al. 2002). The Wnt/Ca^{2+} pathway involves an increase in intracellular Ca^{2+} and is activated by Wnt5a and Wnt11 (Miller, Hocking et al. 1999; Topol, Jiang et al. 2003; Weidinger and Moon 2003). Current downstream pathways are not yet identified (Kuhl, Sheldahl et al. 2000). The Wnt/polarity pathway regulates cellular polarity by controlling cytoskeletal organisation via a yet unidentified pathway involving Dsh and in drosophila, the JNK pathway (Peifer and McEwen 2002). The Wnt-signalling pathway is summarised in Figure 1.9

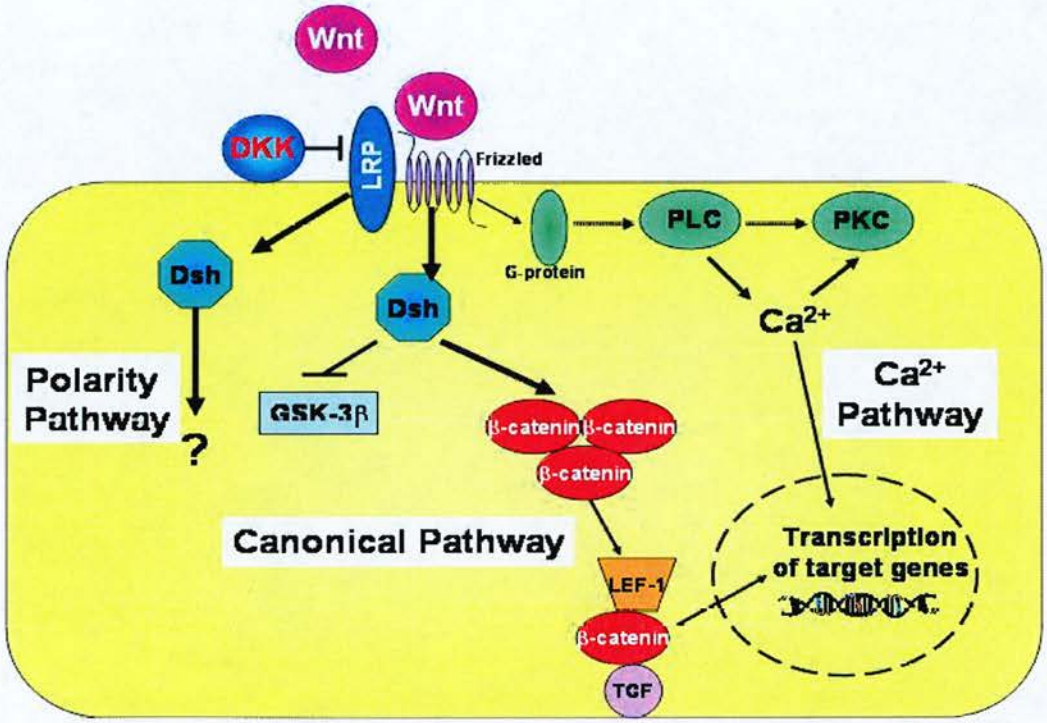


Figure 1.9: The Wnt-signalling pathway. The Wnt ligand binds a Frizzled /low density lipoprotein receptor related protein (LRP) complex, activating the cytoplasmic protein dishevelled (Dsh) Dsh then inhibits the activity of GSK-3β, results in accumulation of β-catenin. β-catenin will then form a complex with TCF and LEF-1, before translocating to the nucleus leading to transcription of Wnt target genes. DKK acts as an antagonist of Wnt-binding to LRPs. The Wnt-Ca^{2+} pathway results in accumulation of Ca^{2+} and the Wnt-polarity pathway is thought to control cytoskeletal organisation by an unidentified pathway. Self prepared.

1.6.2.1 Wnt antagonists

Wnt antagonists are divided into two functional classes, the sFRP class, and the Dickkopf (DKK) class. Members of the sFRP class, which includes the eight known members of the sFRP family, WIF-1 and Cerberus, bind directly to Wnts, thereby altering their ability to bind to the Wnt receptor complex. Members of the DKK class, which comprises the DKK family of proteins, inhibit Wnt signalling by binding to the LRP5/LRP6 component of the Wnt receptor complex. Therefore, theoretically, those antagonists of the sFRP class will inhibit both canonical and noncanonical pathways, whereas those of the DKK class specifically inhibit the canonical pathway.

The DKK family comprises four members (DKK-1 to DKK-4) and a unique DKK-3-related protein named Soggy (Sgy). DKKs contain two characteristic cysteine-rich domains (Cys-1 and Cys-2) separated by a linker region of variable length (Glinka, Wu et al. 1998; Krupnik, Sharp et al. 1999). The most studied member of the DKK family is DKK-1. The characteristic developmental function of DKK-1 is its head-inducing activity (Glinka, Wu et al. 1998). DKK-1 inhibits Wnt-induced stabilisation of β-catenin (Fedi, Bafico et al. 1999) and β-catenin/TCF/LEF-dependent transcription of both artificial and endogenous genes in mammalian and amphibian cells, respectively (Wu, Glinka et al. 2000; Brott and Sokol 2002). However, unlike sFRPs, DKK-1 prevents activation of the Wnt signalling pathway by binding to LRP5/6 rather than to Wnt proteins (Fedi, Bafico et al. 1999; Bafico, Liu et al. 2001; Mao, Wu et al. 2001; Semenov, Tamai et al. 2001). At present, not all Wnts are characterised with regards to their downstream signals and second messengers. Less is known concerning the interaction of Wnt with other signalling pathways.

1.6.2.2 Wnt signalling in the uterus

Several reports indicate that the Wnt pathway is likely to play an important role in murine uterine physiology (Das, Tan et al. 2000; Hou, Tan et al. 2004). A recent study has demonstrated that the Wnt/β-catenin signalling pathway is activated in two distinct stages during implantation in the TCF/Lef-LacZ transgenic mouse (Mohamed, Jonnaert et al. 2005). The authors demonstrated that the Wnt/β-catenin signalling is transiently activated in circular smooth muscle cells to form a banding pattern of activity within the uterus (Mohamed, Jonnaert et al. 2005). Thereafter, the Wnt/β-catenin pathway is activated at the luminal epithelium of prospective implantation sites. Furthermore, this study demonstrates that activation at both sites requires the presence of the blastocyst, which has also been reported to express multiple Wnt genes (Mohamed, Dufort et al. 2004; Mohamed, Jonnaert et al. 2005). The Wnt pathway may also be important in embryo implantation-related events in the human endometrium. High-density cDNA microarray screening of changes in gene expression across the menstrual cycle did not report any change in Wnts or their receptors from the proliferative phase to the mid-secretory phase (Carson, Lagow et

al. 2002; Kao, Tulac et al. 2002); however significant changes were observed in expression of Wnt antagonists with marked increases observed in DKK-1 expression (Tulac, Nayak et al. 2003; Tulac, Overgaard et al. 2006). The transcript encoding DKK was localised to the uterine stroma and is reported to be progesterone-dependent, implying a role in stromal differentiation (Giudice 2004; Tulac, Overgaard et al. 2006).

1.6.3 Cyclic Adenosine Monophosphate (cAMP) and protein kinase (PKA) pathway

As described briefly in section 1.1.4.2 cAMP is generated upon binding of a ligand to a G-protein coupled receptor. Upon ligand binding, the Gα subunit is released from the trimeric Gαβγ complex and regulates the activity of adenylyl cyclase. Two molecules of cAMP bind to the cAMP-dependent PKA (which in its basal state is composed of two regulatory and two catalytic subunits) (Skalhegg and Tasken 2000) and induce a conformational change which results in dissociation and activation of the catalytic subunits. The catalytic subunits may then phosphorylate target molecules in the cytoplasm or diffuse into the nucleus and modulate the activity of transcription factors by phosphorylation (Gellersen and Brosens 2003). Two primary targets of PKA phosphorylation are the cAMP response element binding protein (CREB) and the related cAMP response element (CRE) modulator (CREM) (Mayr and Montminy 2001). The pathway is summarised in Figure 1.10. Phosphorylated CREB binds to CRE sequences in the 5'-flanking region of cAMP-responsive genes (Christian, Mak et al. 2002). Both CREM and CREB require binding to CREB binding protein (CBP) for maximal transcription activity (Fimia, De Cesare et al. 1999; Christian, Mak et al. 2002). Human ESCs express CREB and six CREM isoforms (Gellersen, Kempf et al. 1997). However, decidualisation *in vitro* is not associated with altered expression of CREB or any of the CREM isoforms (Christian, Mak et al. 2002). Moreover, the decidual PRL gene contains a single non-canonical CRE, but mutation of this response element does not impair *in vitro* decidualisation (Telgmann, Maronde et al. 1997). This would suggest that decidualisation requires the induction or modulation of cell-specific PKA-dependent, intermediate factors. One candidate is the CCAAT/enhancer-binding proteins (C/EBP), a group of

conserved phosphorylation site in the kinase-inducible domain (Christian, Mak et al. 2002). Analysis of the decidual prolactin promoter revealed the presence of 20 C/EBP-binding sites, two of which are located within the minimal PKA-responsive region, located between -332 bp and -270 bp relative to the transcription start site (Berwaer, Martial et al. 1994). This region also contains a progesterone response element-half site in close proximity to the two C/EBP-binding sites (Berwaer, Martial et al. 1994). Recently a study has elucidated that a functional association exists between PR and C/EBP to modulate *in vitro* decidualisation of human ESC (Christian, Pohnke et al. 2002).

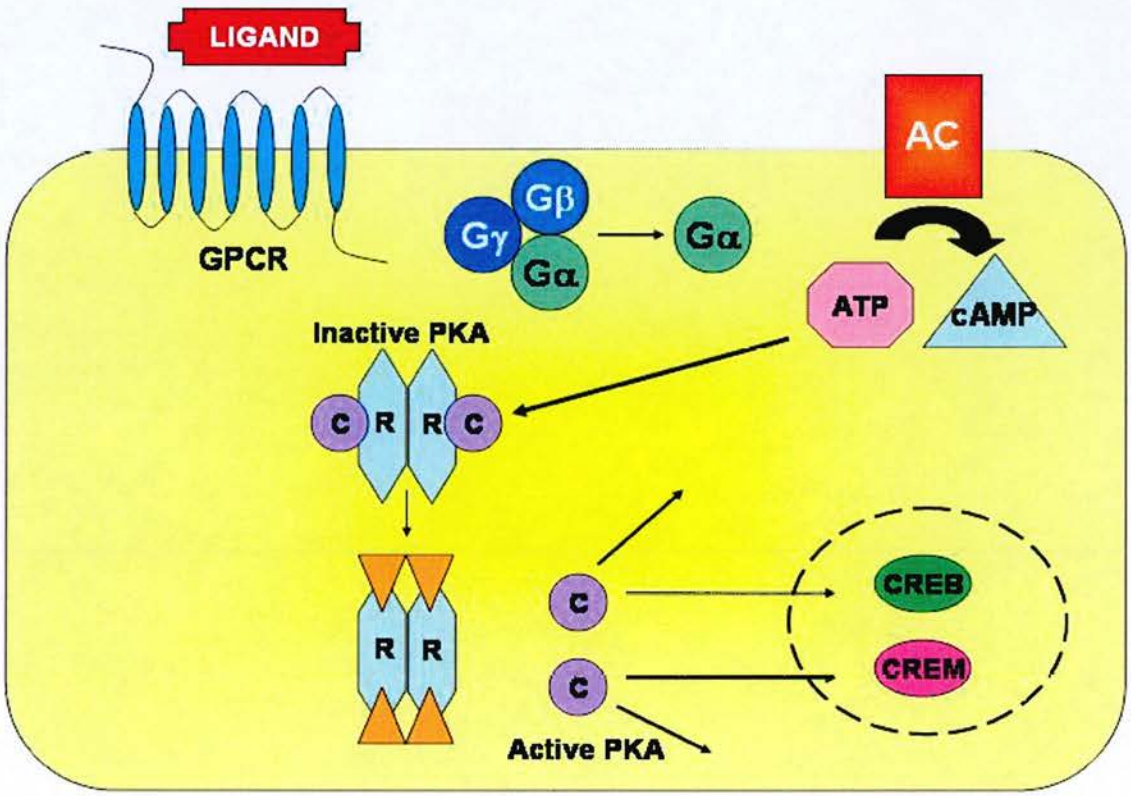


Figure 1.10: The cAMP and PKA pathway. Ligand binding to the G-protein coupled receptor (GPCR) releases the Gα subunit, which regulates the activity of adenylyl cyclase (AC), causing accumulation of cAMP. Active PKA catalytic units (C) are released following cAMP binding to the PKA regulatory subunits (R). The active catalytic subunits phosphorylate targets in both they cytoplasm and the nucleus, such as CREB and CREM. Self prepared.

1.7 Hypothesis

Decidualisation is governed by the steroid hormone progesterone acting via its nuclear receptor (Huang, Tseng et al. 1987; Zhu, Huang et al. 1990; Tseng, Gao et al. 1992; Mizuno, Tanaka et al. 1998; Brosens, Hayashi et al. 1999), but at a paracrine level may be regulated by a myriad of, cytokines and growth factors, including the Wnt-signalling pathway antagonist, Dickkopf (DKK) (Tulac, Nayak et al. 2003; Giudice 2004; Jones, Stoikos et al. 2006; Tulac, Overgaard et al. 2006). TGFβ1 is abundantly expressed in both endometrial epithelial and stromal cells and is thought to play a critical role in cyclic tissue remodelling and inflammatory events associated with menstruation (Bruner, Rodgers et al. 1995; Ulloa, Creemers et al. 2001). I hypothesise that TGFβ1 may affect either PR or DKK to regulate the decidual process, as investigated in Chapter 3. Additionally, I hypothesise that TGFβ1 may suppress the production of decidualisation marker proteins, as investigated in Chapter 4. Decidualisation is also associated with a unique immune environment, characterised by the presence of large numbers of uterine-specific natural killer cells (uNK) (Bulmer, Lunny et al. 1988). uNK cells increase in number in secretory phase endometrial stroma, however, they lack the nuclear progesterone receptor (Henderson, Saunders et al. 2003) and growth and differentiation may therefore depend on interactions with other cell types and regulation by other cytokines (Trundley and Moffett 2004). I hypothesise that uNK cell regulation may involve hormones such as luteinising hormone (LH) and human chorionic gonadotrophin (hCG), as investigated in Chapter 5.

1.8 Aims of this project

The aims of this study were to investigate stromal cell function in non-pregnant endometrium and decidua of early pregnancy. A major focus of these investigations was the role played by TGFβ1 in modulation of the regulators of decidualisation and the decidualisation marker proteins. Initial studies used the ESC as a model for inducing decidualisation *in vitro*. Later the studies used DSC cells as a model for decidualisation in the presence of a blastocyst. The role of hCG on uNK cell function and expansion was also investigated. Experiments in this thesis were designed to address the following questions:

1. As PR is retained in the stroma throughout the cycle and TGFβ1 is activated by uPA at the late secretory phase of the cycle, does TGFβ have any effect on PR?

2. As TGFβ1 is present and in its active form at the time of decidualisation of the stroma, does TGFβ1 have any effect on the process of decidualisation, either by regulating mediators of decidualisation or the decidualisation marker proteins?

3. Does TGFβ1 have any effect on expression of decidualisation markers in stromal cells obtained from 1st trimester decidua?

4. Does TGFβ1 signal via the SMAD signalling pathway in the culture experiments?

5. Are the TGFβ1-induced responses mediated via the SMAD signaling pathway?

6. As the expansion of uNK cell numbers could be related to levels of LH/hCG, does hCG have any effect on uNK cells and do uNK cells possess the LH/hCG receptor?

7. As the macrophage mannose receptor binds sulphated oligosaccharides, could the presence of a mannose receptor on uNK cells facilitate LH/hCG binding?

Chapter 2

2 General Materials and Methods

2.1 Patient selection and recruitment

2.1.1 Tissue Collection, processing of human uterine tissue and ethical approval

Human endometrial tissue specimens (n = 35) were obtained from women undergoing surgery for non-malignant gynaecological conditions (see Table 2.1a, patient numbers 1 – 35). Biopsies were collected with an endometrial suction curette (Pipelle, Laboratoire CCD, Paris, France) or alternatively, full thickness endometrial samples were obtained. These included superficial and basal endometrium plus the endometrial-myometrial junction. All patients were of reproductive age, had regular menstrual cycles between 25-35 days and had not received exogenous hormones or used an intrauterine contraceptive device in the three months prior to surgery. All subjects had a serum sample collected at the time of surgery for the determination of circulating oestradiol (E_2) and progesterone (P) levels by Radio Immunoassay (RIA). All samples were consistent with the designated cycle stage based on standard histological criteria of Noyes et al (Noyes, Hertig et al. 1950) and the patient's reported last menstrual period (Critchley, Henderson et al. 2002) and, circulating E_2 and P levels at time of biopsy collection, see Table 2.1. Endometrial tissue was collected in sterile Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, Poole, Dorset, UK) and processed in one of two ways, either for histology (fixation in 10% neutral buffered formalin (NBF) 24 h at 4°C followed by storage in 70% ethanol prior to wax embedding), or for tissue culture (separation of glandular epithelium and stromal cells, in order to culture the stromal cells).

Decidual tissue specimens were obtained from women who had undergone surgical termination by vacuum aspiration, during the first trimester of pregnancy. All patients were operated on under general anaesthesia (see Table 2.1b, patient numbers 1 – 46).

All women had an ultrasound scan to confirm viability of pregnancy and gestational age. All material from the suction curettage procedure was collected. Decidual tissue (n = 46) was selected by macroscopic inspection from the products of the termination aspiration procedure. Sterile gauze was held taut over a waste collection vessel and the contents of the tissue collection container poured over the gauze. Excess blood/saline was removed by gentle compression. Sterile forceps were used to extract the pieces of decidua parietalis (away from the site of implantation). Identification was aided by gently washing the tissue in sterile saline. In this way it is possible to distinguish between decidua parietalis, decidua basalis and trophoblast. Trophoblast villi are readily identifiable by the light pink colour and frond-like appearance of the tissue. Decidua parietalis is generally grey in appearance and quite smooth and solid in consistency. Decidua basalis is distinguishable by its ragged, yellowish appearance and often contains haemorrhagic areas. This procedure requires two people and was performed by myself and a trained Research Sister.

Thereafter, decidual parietalis tissue was collected in sterile RPMI 1640 medium (Sigma) in preparation for the uterine Natural Killer cell separation procedure (detailed in 4.2.1.2). A 1 cm^3 section of tissue was placed in "RNA Later" (Ambion Europe Ltd., Cambridgeshire, UK), and stored at 4°C for a maximum of 2 months prior to mRNA extraction (section 2.3.1). A second 1 cm^3 section of tissue was placed into 10 % NBF, fixed for 24 h then placed in 70 % ethanol prior to paraffin wax embedding. Decidua was only collected from women with a pregnancy of gestational age between 7 and 12 weeks. All work on human samples was performed in a designated containment level one tissue culture room within class II Microbiological Safety Cabinets. Due to the potential presence of infectious pathogens both the research nurse and I were immunised and shown to be protected against Hepatitis B.

Informed patient consent was obtained in writing from each patient prior to tissue collection, by a dedicated research nurse, after local ethical committee approval for the study was granted (Appendix 1).

Table 2.1: Patient sample details

Table 2.1a: Patient sample details for human endometrial tissue specimens.

Patient number	Date of Collection	Age	Day of Cycle	Blood E_2 (pg/ml)	Blood P (ng/ml)	Ethics No	Gynaecological procedure	Form of Endometrial Biopsy	Presenting gynaecologica Symptoms
1	11/06/2000	49	20	608	34.9	17	Hysteroscopy / Insert Mirena	Pipelle	Pelvic Pain
2	14/11/2000	51	20	75	2.1	17	Total Abdominal Hysterectomy/ bilateral Salpingo	Pipelle	Fibroids
3	07/04/2001	37	6	215	1.8	17	Diagnostic Laparoscopy	Pipelle	Iliac Fossa Pain
4	07/09/2001	33	11	373	1.8	17	Diagnostic Laparoscopy	Pipelle	Ovarian cyst
5	18/7/2001	39	13	964	3.9	17	Diagnostic Laparoscopy	Pipelle	Dysmenorrhoea
6	09/10/2001	37	7	234	1.7	17	Laparoscopic Sterilisation	Pipelle	Fertility
7	10/08/2001	33	6	255	2.8	17	Diagnostic Laparoscopy	Pipelle	Iliac Fossa Pain

Patient number	Date of Collection	Age	Day of Cycle	Blood E2 (pg/ml)	Blood P (ng/ml)	Ethics No	Gynaecological procedure	Form of Endometrial Biopsy	Presenting gynaecologica Symptoms
8	11/06/2001	44	13	359	1.8	17	Uterine Baloon Therapy	Pipelle	Fibroids/ Menorrhagia/ Dysmenorrhoea
9	16/4/2002	41	31	650	51	17	Total Abdominal Hysterectomy	Wedge	Fibroids/ Menorrhagia/ Dysmenorrhoea
10	25/4/2002	44	25	276	42.1	17	Total Abdominal Hysterectomy/ bilateral Salpingo	Wedge	Fibroids/ Menorrhagia/ Dysmenorrhoea
11	25/4/2002	39	10	835	10.14	17	Laparoscopic Sterilisation	Pipelle	Fertility
12	05/01/2002	42	14	478	23.66	17	Total Abdominal Hysterectomy	Pipelle	Fibroids/ Menorrhagia/ Dysmenorrhoea/ pain mid-cycle
13	05/08/2002	41	9	1002	13.68	17	Total Abdominal Hysterectomy	Pipelle	Fibroids/ Dysmenorrhoea
14	14/5/2002	42	18	401	72.28	17	Total Abdominal Hysterectomy	Pipelle	Fibroids/ Menorrhagia

Patient number	Date of Collection	Age	Day of Cycle	Blood E2 (pg/ml)	Blood P (ng/ml)	Ethics No	Gynaecological procedure	Form of Endometrial Biopsy	Presenting gynaecological Symptoms
15	26/8/2002	37	23	745	127.2	17	Hysteroscopy Insert Mirena	Pipelle	Menorrhagia/ Dysmenorrhoea
16	30/9/2002	48	20	552	78.9	17	Total Abdominal Hysterectomy/ bilateral Salpingo	Wedge	Menorrhagia/ Ovarian cyst
17	13/1/2003	37	10	NA	NA	17	Diagnostic Laparoscopy	Pipelle	Menorrhagia/ Dysmenorrhoea/ daily pain/ Endometriosis
18	27/1/2003	38	10	353	63	17	Hysteroscopy / Dilation and Curettage	Pipelle	Menorrhagia/ Dysmenorrhoea/ Intra-menstrual bleeding and pain
19	27/1/2003	21	16	589	20.82	17	Diagnostic Laparoscopy	Pipelle	Menorrhagia/ Dysmenorrhoea/ daily pain/ Endometriosis
20	03/10/2003	35	~ 27	398	63	17	NA	Pipelle	Vulval disorder
21	22/9/2003	47	11	1796	2.32	17	Insert Mirena	Pipelle	Dysmenorrhoea

Patient number	Date of Collection	Age	Day of Cycle	Blood E2 (pg/ml)	Blood P (ng/ml)	Ethics No	Gynaecological procedure	Form of Endometrial Biopsy	Presenting gynaecological Symptoms
22	12/01/2003	46	16	574	4.44	40	Hysteroscopy / Insert Mirena	Pipelle	Polyps/ Menorrhagia/ Intra-menstrual Bleeding
23	19/7/2004	30	4	242	6.15	40	Diagnostic Laparoscopy	Pipelle	Dysmenorrhoea
24	23/8/2004	43	8	581	68.94	17	Hysteroscopy / Dilation and Curettage / Polypectomy	Pipelle	Fibroids/ Polyps/ Menorrhagia/ Intra-menstrual Bleeding
25	23/8/2004	32	12	1651	4.04	17	Diagnostic Laparoscopy / Diathermy to Endometriosis	Pipelle	Menorrhagia/ Persistant Pain
26	25/8/2004	33	22	520	62.68	17	Diagnostic Laparoscopy	Pipelle	Dyspareunia/ Ovarian cyst
27	09/07/2004	40	12	667.03	10.86	17	Total Abdominal Hysterectomy	Wedge	Fibroids/ Menorrhagia/ Dysmenorrhoea
28	11/01/2004	43	16	607.2	146.41	17	Hysteroscopy / Endometrial Sampling	Pipelle	Menorrhagia/ Dysmenorrhoea/ Daily Spotting

Patient number	Date of Collection	Age	Day of Cycle	Blood E2 (pg/ml)	Blood P (ng/ml)	Ethics No	Gynaecological procedure	Form of Endometrial Biopsy	Presenting gynaecological Symptoms
29	02/01/2005	42	8	208.52	0.88	17	Microwave Endometrial Ablation	Pipelle	Menorrhagia
30	02/02/2005	39	22	516.89	93.23	73	Laparoscopic Sterilisation	Pipelle	Fertility
31	03/02/2005	46	15	641.34	2.5	17	Hysteroscopy / Insert Mirena	Pipelle	Menorrhagia
32	17/5/05	35	9	278.52	2.45	17	Hysteroscopy / Dilation and Curettage	Pipelle	Polyps/ Menorrhagia/ Intra-menstrual Bleeding
33	06/06/2005	47	26	113.18	60.4	17	Laparotomy / Ovarian Cystectomy	Pipelle	Ovarian cyst/ Pain
34	22/6/05	30	~ 27	166.18	7.61	17	/ Insert IUCD	Pipelle	Pain
35	10/04/2005	45	~ 27	323.11	27.3	17	Hysteroscopy / Insert Mirena	Pipelle	Fibroids/ Menorrhagia/ Dysmenorrhoea

Table 2.1a: Patient sample details for human endometrial tissue specimens. n = 35 endometrial samples.

Table 2.1b: Patient sample details for human 1st trimester decidual tissue specimens

Patient number	Date of Collection	Age	PARITY LB	PARITY MT	Gestation (Wks + day)	Ethics no	Gynaecological procedure
1	14/04/2004	28	1	0	8+4	73	STOP (with Miso)
2	01/11/2004	23	0	0	9+3	73	STOP (with Miso)
3	10/11/2004	20	1	0	7+5	73	STOP (with Miso)
4	15/11/2004	36	3	0	8+3	73	STOP (with Miso)
5	15/11/2004	31	0	0	7+6	73	STOP (with Miso)
6	24/11/2004	36	1	0	7+6	73	STOP (with Miso)
7	24/11/2004	23	0	1	8+4	73	STOP (with Miso)
8	29/11/2004	30	2	2	8+3	73	STOP (with Miso)
9	01/12/2004	23	2	0	7+6	73	STOP (with Miso)
10	25/01/2005	24	2	1	11	73	STOP (with Miso)

Patient number	Date of Collection	Age	PARITY LB	PARITY MT	Gestation (Wks + day)	Ethics no	Gynaecologica procedure
11	13/09/2005	26	2	0	8	05/S1104/12	STOP (with Miso)
12	13/09/2005	21	0	0	8	05/S1104/12	STOP (with Miso)
13	14/09/2005	43	2	1	7+4	05/S1104/12	STOP (with Miso)
14	27/09/2005	26	0	0	8+6	05/S1104/12	STOP (with Miso)
15	27/09/2005	21	0	0	9+5	05/S1104/12	STOP (with Miso)
16	28/09/2005	43	0	1	8	05/S1104/12	STOP (with Miso)
17	28/09/2005	26	1	0	9+2	05/S1104/12	STOP (with Miso)
18	29/09/2005	23	0	0	8+5	05/S1104/12	STOP (with Miso)
19	29/09/2005	21	0	0	9+1	05/S1104/12	STOP (with Miso)
20	04/10/2005	30	0	0	9+3	05/S1104/12	STOP (with Miso)

Patient number	Date of Collection	Age	PARITY LB	PARITY MT	Gestation (Wks + day)	Ethics no	Gynaecologica procedure
21	04/10/2005	18	1	1	8+3	05/S1104/12	STOP (with Miso)
22	12/10/2005	26	2	0	9+3	05/S1104/12	STOP (with Miso)
23	12/10/2005	20	1	4	9+6	05/S1104/12	STOP (with Miso)
24	26/10/2005	24	0	0	11+1	05/S1104/12	STOP (with Miso)
25	28/11/2005	30	3	1	10+2	05/S1104/12	STOP (with Miso)
26	29/11/2005	24	0	0	11	05/S1104/12	STOP (with Miso)
27	02/12/2005	27	1	1	10+3	05/S1104/12	STOP (with Miso)
28	02/12/2005	28	3	0	8+2	05/S1104/12	STOP (with Miso)
29	05/12/2005	23	1	1	10+4	05/S1104/12	STOP (with Miso)
30	13/12/2005	24	0	0	9+5	05/S1104/12	STOP (with Miso)

Patient number	Date of Collection	Age	PARITY LB	PARITY MT	Gestation (Wks + day)	Ethics no	Gynaecologic procedure
31	16/02/2006	36	0	0	10+3	05/S1104/12	STOP (with Miso)
32	21/02/06	21	0	0	8+1	05/S1104/12	STOP (with Miso)
33	22/02/06	19	0	1	10+2	05/S1104/12	STOP (with Miso)
34	02/03/06	25	0	0	10	05/S1104/12	STOP (with Miso)
35	02/03/06	43	2	0	9+2	05/S1104/12	STOP (with Miso)
36	02/03/06	18	1	0	9+6	05/S1104/12	STOP (with Miso)
37	09/03/06	19	0	0	11	05/S1104/12	STOP (with Miso)
38	15/03/06	25	0	0	10+4	05/S1104/12	STOP (with Miso)
39	15/03/06	25	0	0	< 8	05/S1104/12	STOP (with Miso)
40	20/03/06	20	0	0	10+1	05/S1104/12	STOP (with Miso)

Patient number	Date of Collection	Age	PARITY LB	PARITY MT	Gestation (Wks + day)	Ethics no	Gynaecologica procedure
41	20/03/06	42	1	0	8+6	05/S1104/ 12	STOP (with Miso)
42	22/03/06	20	0	0	9+2	05/S1104/ 12	STOP (with Miso)
43	22/03/06	34	0	0	8	05/S1104/ 12	STOP (with Miso)
44	22/03/06	30	1	1	9+6	05/S1104/ 12	STOP (with Miso)
45	22/03/06	18	0	0	8	05/S1104/ 12	STOP (with Miso)
46	22/03/06	26	2	0	9+5	05/S1104/ 12	STOP (with Miso)

Table 2.1b: Patient sample details for human 1st trimester decidual tissue specimens. LB = Live births, MT = Miscarriages/Terminations, STOP = surgical termination of pregnancy, Miso = Misoprostol. n = 46 samples

2.2 Cell separation and culture

Endometrial specimens (n = 35) were separated into epithelial and stromal cell preparations after enzymatic digestion as follows. Samples were washed in Dulbecco's phosphate buffered saline (PBS) (Sigma) and minced into 1 mm^3 pieces. The minced tissue was digested in collagenase (1 mg/ml; Sigma) and DNAase (0.1 mg/ml; Sigma) for 80 min at 37°C. Repeat passage through an 18 gauge needle was used to aid tissue dispersion. Tissue was resuspended in 10 ml of RPMI 1640 medium (Sigma). The stromal and glandular epithelium cells were pelleted by centrifugation (700 g, 3 min). The cells were then resuspended in 10 ml of RPMI 1640 medium (Sigma) supplemented with 10 % foetal calf serum (FCS) (Mycoplex, PAA Laboratories, Kingston-Upon-Thames, UK), penicillin (50 μg/ml; Sigma), streptomycin (50 μg/ml; Sigma) and gentamycin (5 μg/ml; Sigma) and dispersed endometrial stromal cells separated from endometrial glands by filtration through a 73 μm nylon sieve (Falcon, VWR International Ltd, Leicestershire, UK). The filtrate, containing the stromal cells, was plated in 75 cm^3 culture flasks (Corning Incorporated, Corning, NY) for a minimum period of 5 days and allowed to reach confluence in the presence of E_2 (0.1 μM; Sigma), 6α-Methyl-17α-acetoxyprogesterone (MPA) (1 μM; Sigma) and basic fibroblast growth factor (bFGF) (5 ng/ml; Peprotec EC Ltd, London, UK) at 37°C in a humidified atmosphere of 5 % CO_2. These supplements were used to provide optimum conditions for ESC growth (Irwin, Utian et al. 1991).

A Fluorescence Activated Cell Sorter (FACS) and immunohistochemical staining for fibroblast markers were used to verify the purity of cells separated by this technique (see section 2.7).

The cells were passaged every 3-4 days by trypsinisation. Media was aspirated from the cells and discarded; cells were washed with 5 ml PBS (Sigma) to remove any residual traces of media. PBS was removed by aspiration and the appropriate volume of Trypsin/EDTA (PAA) added per flask (see Table 2.2). The cells were incubated at 37°C for 3-5 min until they had detached from the flask surface; thereafter the

appropriate volume of RPMI 1640 medium (Sigma) supplemented with 10 % foetal calf serum (FCS) (Mycoplex), penicillin (50 μg/ml; Sigma), streptomycin (50 μg/ml; Sigma) and gentamycin (5 μg/ml; Sigma) was added to prevent any further action of trypsin/EDTA.

Once passaged, cells were seeded at a concentration of 2 x 10^5 cells/ml in 6-well-plates (Corning Inc, Costar, Sigma) or 2 x 10^5 cells/ml in 2-well chamber slides (LabTec, VWR International Ltd) and incubated for 24 h to allow cell adherence. The cells were cultured until they were 90 % confluent. 24 h prior to commencement of treatment with test agents, the culture medium was changed to serum-free media or 2 % FCS RPMI 1640 for *in vitro* decidualisation. Two separate ESC culture experiments were designed. In the first, the cells were treated with serum-free RPMI 1640 medium that contained test agents, for example, E_2 (0.1 μM; Sigma), and/or MPA (1 μM; Sigma) and/or TGFβ1 (10 ng/ml; R&D Systems, Abington, UK). In the second culture experiment, decidualisation of the cells was induced with RPMI 1640 medium containing 2 % FCS, 8-Bromoadenosine 3',5'-cyclic monophosphate sodium salt (8-Br-cAMP) (0.5 mM; Sigma) and MPA (1 μM), ('decidualising medium' - DM), for 6 days, thereafter, the cells were treated with RPMI 1640 2 % FCS and DM containing TGFβ1. Cells were maintained under these conditions for up to 72 h.

T47D cells, the breast cancer epithelial cell line, known to constitutively over express PR A and PR B (Horwitz, Mockus et al. 1982) and the recently identified PR C (Wei, Gonzalez-Aller et al. 1990), were cultured in RPMI 1640 medium (Sigma) supplemented with 10 % FCS (Mycoplex), penicillin (50 μg/ml; Sigma), streptomycin (50 μg/ml; Sigma) and gentamycin (5 μg/ml; Sigma) in the presence of insulin-transferrin-sodium (5 μg/ml; Sigma), and selenite media supplement (5 ng/ml; Sigma) (ITS). The cells were treated with serum-free RPMI 1640 medium supplemented with ITS (Sigma) that contained test agents, for example, oestradiol (0.1 μM; Sigma), and/or MPA (1 μM; Sigma) and/or TGFβ1 (10 ng/ml; R&D Systems). Cells were maintained under these conditions for up to 72 h.

Flask size (cm^2)	Volume of Trypsin/EDTA to be added (ml)	Volume of RPMI to be added (ml)
75	1	9
150	1.5	8.5
225	2	8

Table 2.2: Appropriate volumes of Trypsin/EDTA and RPMI to be added for passaging of cells.

2.3 RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

2.3.1 RNA Extraction

The supernatant from the treated cell cultures was aspirated, retained and either frozen at –20°C or maintained at 4°C if being used immediately. Cells were washed in PBS twice to remove any residual supernatant. Total RNA was isolated from the cultured endometrial stromal cells using a guanidinium thiocyanate-based extraction reagent, "Total RNA Isolation Reagent" (TRIR) (Abgene, Surrey, UK); 1 ml of TRIR was added to each well of a 6-well plate. Cells were left for 5 min to facilitate cell membrane rupture. The contents of the wells were then transferred to "phase lock gel" tubes (Eppendorf, Hamburg, Germany) and kept on ice for the remaining extraction. Chloroform (200 μl/ml) was added and the tubes shaken vigorously for several seconds. The tubes were centrifuged in a pre-cooled, refrigerated centrifuge at 4°C, 12000 g for 20 min. Upon centrifugation the phase lock gel migrates to form a tight seal between phases of an aqueous/organic extraction. The upper aqueous phase above the phase lock gel contained RNA with the organic phase, containing DNA and protein, trapped beneath the gel layer thus allowing easy isolation of the aqueous phase. The aqueous layer was subsequently poured into labelled 1.5 ml Eppendorf microcentrifuge tubes. An equal volume of isopropanol was added to each sample and mixed by inverting the tubes. The mixture was left on ice for 1 h to allow for RNA precipitation. The samples were centrifuged in a pre-cooled, refrigerated

centrifuge at 4°C, 12000 g for 15 min to pellet the RNA. The supernatant was removed using a sterile Pasteur pipette and the pellet was washed with 500 µl of 70 % ethanol. After gentle mixing of the solution by inverting the tubes, the samples were centrifuged for 5 min in a pre-cooled, refrigerated centrifuge at 4°C, 12000 g. The ethanol was removed from each tube and 20-50 µl RNA Storage Buffer (RSB) (Ambion, Austin, TX, USA) was added depending on the size of the mRNA pellet. Samples were dissolved in RSB and subsequently frozen at -80°C.

The concentration of total RNA in the samples was measured on a GeneQuant spectrophotometer (WPA, Cambridge, UK). Each sample was vortexed and 1 µl removed, which in turn was diluted in 9µl distilled water. All tubes were thoroughly mixed before measuring the wavelength at both 260 nM and 280 nM. A blank used to zero the machine consisted of 1 µl RSB diluted in 9 µl of distilled water. All samples were diluted in diethylpyrocarbonate-treated water (DEPC water) to a concentration of 100 ng/µl.

2.3.2 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA samples (at 100 ng/µl) were reversed transcribed using a multiscribe reverse transcriptase (1.25 IU/µl), random hexamers (2.5 µmol/l), $MgCl_2$ (5.5 mmol/l), dNTPs (1 mmol/l) and an RNAase inhibitor (0.4 IU/µl) (PE Biosystems, Warrington, UK). All reactions consisted of 16 µl of reverse transcription mix per tube and 4 µl of mRNA. Once the contents of the tube were mixed, 50 µl of mineral oil was added to prevent evaporation of the reaction mix during the reverse transcription process. Samples were incubated for 20 min at 25°C, 60 min at 42°C and then at 95°C for 5 min (Omnigene PCR machine, UK). The resulting cDNA was diluted 2.5x with TE buffer (10 mmol/l Tris pH 8.0 and 1 mmol/l EDTA in DEPC water) and stored at 4°C.

The precision of the reverse transcription (RT) reaction was previously calculated by a colleague, Elena Faccenda (laboratory research support). An mRNA sample was

taken and 8 RT reactions set up in 8 separate tubes. These were compared in a single PCR run on one primer and probe set. The precision was found to be 3.6%.

2.4 Polymerase Chain Reaction (PCR)

2.4.1 Semi-quantitative PCR (PCR)

Polymerase chain reaction (PCR) was used for the amplification of a specific part of a DNA molecule, which lies between two regions of known sequence, using a DNA polymerase enzyme that is tolerant to elevated temperatures. The oligonucleotides act as primers for the DNA polymerase and the denatured strands of the cDNA acts as the template. Primers were designed individually for each DNA target sequence using the Primer Express computer program. A reaction mix containing the reaction buffer, 2X BioMix; (125 mM Tris-HCL (pH 8.8 at 25°C), 32 mM $(NH_4)2SO_4$, 3 mM $MgCl_2$ (final concentration is 1.5 mM), 0.02 % Tween 20, 2 mM deoxyribonucleotides (dNTPs), a stabiliser and an inert dye (BioMix Red, Bioline Ltd. London, UK) the cDNA, and 0.5 μM of the primers specific for the gene of interest (see Table 2.3) and a heat-stable polymerase, BIOTAQ DNA Polymerase (Bioline Ltd, London, UK) was made up to 20 μl with sterile, nuclease-free water.

In a thermal reactor (Omnigene bioproducts, Cambridge, MA) the DNA was denatured at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, the annealing temperature specific for each primer pair (Table 2.3) for 30 s and 72°C for 1 min, and finally 72°C for 10 min. The PCR reaction products obtained were run on a 2 % agarose gel with ethidium bromide, a dye that binds to double stranded DNA by intercalations between the base pairs and fluoresces when irradiated in the UV part of the spectrum. The products were visualised by UV illumination and subsequently photographed. A DNA marker (Bioline Ltd) was run in an adjacent lane to allow identification of the size of DNA fragments detected.

Amplicon	Accession Number	Forward Primer	Reverse Primer	Annealing Temp °C	Product size (bp)
GAPDH	NM_002046	CTGCACCACC AACTGCTTAG C	ATGCCAGTG AGCTTCCGT TC	58	205
LH/hCG receptor	NM_000233	ATGAAGCAG CGGTTCTCG	TTGACAGGG AGGTAGGCA AG	62	203
MMR1	X55635	CTACCCCTGC TCCTGGTTTT T	TGAAACACT CATAATCTG AGATTC	60	203
TGFβ1 R1	NM_004612	ACTATCGTAA AGTCATCACC TGGC	TCCTCTTCAT TTGGCACTC GA	59	150
SMAD 4	NM_005359	TCCTCATGTG ATCTATGCCC	TGCAGTGTT AATCCTGAG AG	54	176

Table 2.3: Primers for PCR. All were designed by Dr Elena Faccenda, a member of the laboratory group.

2.4.2 Quantitative Real-time PCR (Q-RT-PCR)

Quantitative Real-time-PCR provides an accurate method for determination of levels of a gene-specific sequence of complimentary DNA (cDNA). This procedure of quantitative measurement is based on detection of a fluorescent signal produced proportionally during amplification of a PCR product. Primers and probes were designed individually for each DNA target sequence using the Primer Express computer program (see Table 2.4). Q-RT-PCR is characterised by the choice of a relatively short sequence to allow maximum efficiency of amplification. The probe is designed to anneal to the target sequence between the forward and reverse primers. The probe is labelled at the 5' end with a reporter fluorochrome (FAM; 6-

carboxyfluorescein) and a quencher fluorochrome (TAMRA; 6 carboxytetramethylrhodamine) added at 3' end. The probe is designed to have a higher T_m than the primers. In the intact state the quencher, suppresses all fluorescence by the reporter because of its proximity. If the target sequence is present the probe anneals to the cDNA in between the forward and reverse primer sites and this is referred to as the polymerisation step. When the target sequence is amplified during a PCR reaction the probe is cleaved by AmpliTaq Gold (Applied Biosystems, Warrington, UK) (which has Y shape structure and polymerisation dependent 5'-3' nuclease activity (Holland, Abramson et al. 1991)). This results in separation of the reporter dye from the quencher dye so that the quencher is no longer capable of suppressing the reporter fluorescence with the result being an increase in fluorescence. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle and can be measured directly (Figure 2.1). Fluorescence is detected only if the target sequence for the probe is amplified during the reaction thus preventing the detection of non-specific amplification.

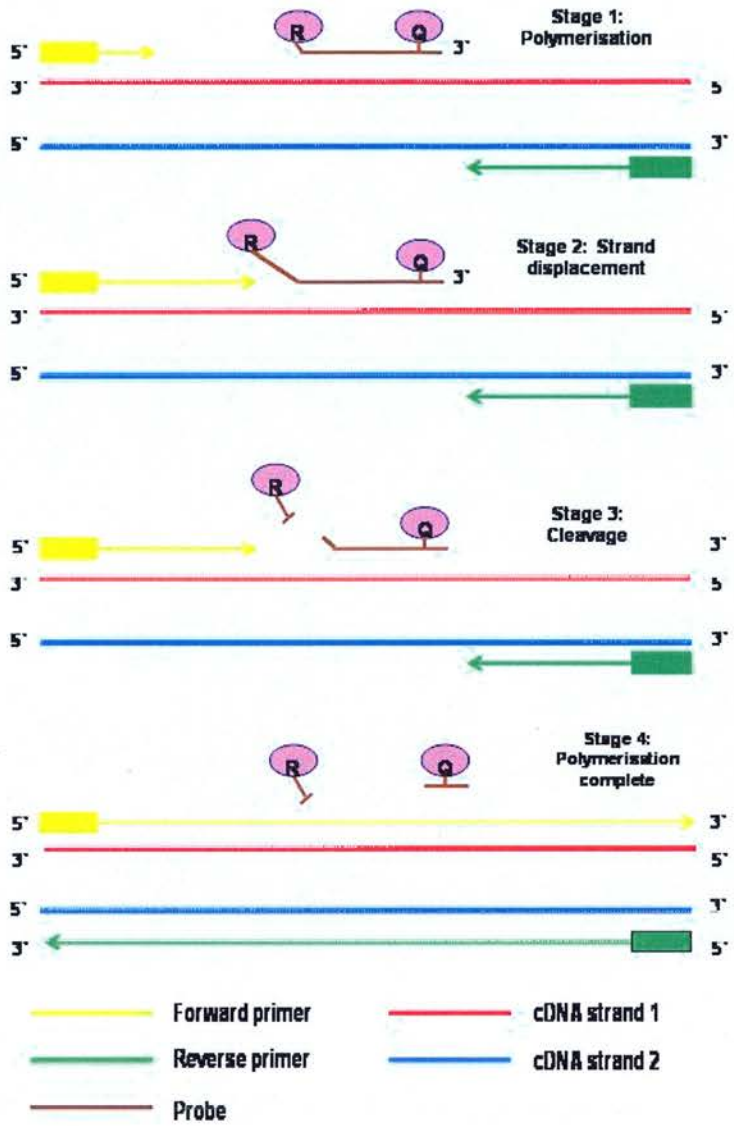


Figure 2.1: Diagrammatic representation of Q-RT-PCR process. R = Reporter Dye and Q = Quencher. Adapted from Taqman PCR Reagent Kit Protocol.

Acquisition of data occurs when PCR amplification is still in the exponential phase. This is the cycle number when the reporter dye emission intensities rises above an arbitrary threshold value (background noise); this cycle number is the threshold cycle (C_t). The 18S is a measure of the ribosomal RNA content of the sample and as 18S levels remain relatively constant in cells this allows corrections to be made for the total amount of RNA protein in the individual samples. The ΔC_t is the difference between the FAM C_t and the 18S C_t and enables the amplified signal to be normalised against the total RNA content. The mean ΔC_t between the duplicates on the PCR plate was calculated. This was then used to calculate the $\Delta\Delta C_t$ which relates the amount of cDNA of the specific amplicon to the ΔC_t value and the control cDNA in that experiment. Within each experiment the ΔC_t was related to its own control. The $2^{-\Delta\Delta Ct}$ is then calculated which shows the fold increase or decrease in mRNA expression of the samples in relation to their control. The control having a $2^{-\Delta\Delta Ct}$ of 1.

Primers were diluted to 250 μM and probes to 50 μM in TE buffer (10 mM Tris; 1 mM EDTA in DEPC H_2O). PCR reaction mixtures contained TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems) (7.2 mM $MgCl_2$; 1.6 mM Stratagene dNTP mix; 1.6 mM Boehringer dNTP mix; 0.05 U/μl Taq Polymerase; 2x PCR buffer and 0.06 % reference dye diluted in DEPC H_2O) and specific forward and reverse primers (250 nM; Biosource, Nivelles, Belgium) and probe (50 nM; Biosource) in a final volume of 25 μl/well. Ribosomal 18S primers and probe (PE Biosystems, Warrington, UK) were added at a final concentration of at 50 nM. PCR reactions were run on an ABI Prism 7900 (Applied Biosystems). For each sample, a tube containing 45 μl PCR Master Mix and 5 μl cDNA was mixed and 23 μl pipetted into two separate wells on the PCR plate. Two wells containing 5μl of DEPC H_2O in place of cDNA were added to each run to serve as a negative control. Samples were measured and mean values used in subsequent analysis.

Amplicon	Accession Number	Forward Primer	Reverse Primer	Probe
β-actin	X00351	TCACCCACA CTGTGCCCA TCTACGA	CAGCGGAAC CGCTCATTG CCAATGG	ATGCCCCCCC CATGCCATCC TGCGT
IFNγ	X13274	CCAACGCA AAGCAATA CATGA	TTTTCGCTT CCCTGTTTT AGCT	CTCATCCAA GTGATGGCT GAACTGTCGC
IGFBP-1	M59316	CACAGGAG ACATCAGG AGAAGAAA	ACACTGTCT GCTGTGATA AAATCCAT	TTCCAAATTT TACCTGCCAA ACTGCAACAA
IL-10	M57627	CTACGGCGC TGTCATCGA T	TGGAGCTT ATTAAAGG CATTCTTC A	CTTCCCTGTGA AAACAAGAGC AAGGCC
MMR	X55635	TGCGACAGT AAACGAGG CTACA	TCGTTGCT GGAGGATT AGTCAA	ATGCCAGACAC GATCCGACCCT TC
Progesterone Receptor (nuclear)	NM_000926	CAGTGGGCG TTCCAAATGA	GGTGGAATC AACTGTATG TCTTGA	AGCCAAGCCCTA AGCCAGAGATTC ACTTT
Prolactin	NM_000948	GCCCCGGAG GCTATCCTA	TCAGCTCCA TGCCCTCTA GAA	CCAAAGCTGAGA GATTCAGGAGCA AACCA

DKK	AB020315	GGAATAAGT ACCAGACCA TTGACAAC	GGGACTAGC CAGTACTCA TCA	ACCAGCCGTA CCCGTGCGCA
SMA	NM_001100	GTGTTGCCCC TGAAGAGCA T	ATTTTCTCC CGGTTGGCC TT	ACCCTGCTCACG GAGGCACCCCT
TNFα	X02910	GGAGAAGG GTGACCGAC TCA	TGCCCAGA CTCGGCAA AG	CGCTGAGATCA ATCGGCCCGAC TA
Tissue Factor	NM_001993	CACCGACGA GCTTGTGAA GGA	CCCTGCCGG GTAGGAGA A	TGAAGCAGAC GTACTTGGCACG GGT

Table 2.4: Taqman primers and probes. All were designed by Dr Elena Faccenda, a member of the laboratory group.

2.5 Transient transfection and promoter/reporter studies

2.5.1 Reporter constructs and expression vectors

All promoter constructs were cloned in pGL3-Basic (Promega Corp, Southampton, UK). The reporter vectors: dPRL-3000/luc3, dPRL-913/luc3, dPRL-601/luc3, dPRL-332/luc,3 and dPRL-32/luc 3 carrying 3000, 913, 601, 332 and 32 bp, respectively, of 5'-flanking DNA to the decidual-specific promoter of the hPRL gene, were provided by Dr. Birgit Gellersen (Hamburg, Germany). The PRE/-32/luc3 and ERE-tk/Luc3 vectors were obtained from Dr Jan Brosens (Imperial College London, UK). The ARE and GRE share the same sequence as PRE. PCH110, β-galactosidase expression vector, was purchased from Pharmacia Biotech (Piscataway, NJ, USA). Non-targeting siRNA and siRNA specific for SMAD 4 was purchased from Dharmacon (Dharmacon, Perbio Science, Belgium).

2.5.2 Calcium phosphate transfection of primary ESC

Calcium Phosphate co-precipitation was routinely used for transient transfection of ESC (Graham and van der Eb 1973). First passage ESC cells were plated in 24-well-plates in 0.5 ml DCC media, a 1:1 mixture of DMEM and Ham's F-12 containing 10 % FCS that had been depleted of steroids by treatment with dextran-coated charcoal, 100 U/ml penicillin, and 100 μg/ml streptomycin, and supplemented with 10^{-9} M 17ß- E_2 and 1 μg/ml insulin, and incubated at 37°C. The media was changed to 2 % FCS DCC the night prior to transfection then changed again to 5 % FCS DCC 1 h prior to transfection. For each transfection, the DNA (500 μg/well reporter, 200 μg/well expression vector and 50 μg/well PCH110) and the 2×HEPES-buffered saline (Promega Corp) were prepared in separate sterile tubes. Nuclease-free water (Promega Corp) was added to the DNA plasmid and subsequently mixed by vortexing. The calcium chloride ($CaCl_2$) (Promega Corp) was added and the solution mixed again. The solution was allowed to stand for 5 min before vortexing. Working in a class II Microbiological Safety Cabinet, the tube containing 2× HEPES-buffered saline was gently vortexed with the cap off to accommodate the addition of the prepared DNA solution. DNA was added to the transfection mixture in a "dropwise" fashion. Calcium phosphate precipitates were readily formed in the transfection mixture. DNA was readily absorbed onto the precipitate, thereby changing the characteristics of the particle. When complete, the solution appeared slightly opaque due to formation of calcium phosphate-DNA co-precipitates. The tube containing transfection mix was incubated at room temperature for 30 min, after which it was finally vortexed prior to cell transfection. Transfection solution (120 μl/well) was added in a dropwise fashion in order to evenly distribute the precipitate over the cells and the plates were incubated at 37°C. The calcium phosphate- DNA co-precipitates were taken up by the cells, via endocytosis or phagocytosis, within 1 h of contact. The culture medium was changed 4-6 h after the addition of the transfection mixture and replaced with 0.5 ml 2 % FCS DCC with or without the following treatments; 8-Br-cAMP (0.5 mM), MPA (1 μM), dexamethasone (DEX, 0.25 μM, Sigma), dihydrotestosterone (DHT, 0.1 μM, Sigma), and TGFβ1 (10

ng/ml). Cells were incubated for a further 48 h after which the cell extracts were harvested and assayed as below. Each transfection was performed in triplicate.

2.5.3 Promoter/reporter assay linked to luciferase

The chemical energy of Luciferin oxidation is converted through electron transition, forming the product molecule oxyluciferin, thereby producing light which is quantitatively read by a luminometer. The cell lysates were prepared by removing the culture media from the cell by careful aspiration to avoid dislodging adherent cells. The cells were subsequently washed with 0.5 ml of PBS prior to addition of 50 µl of 1 × reporter lysis buffer (RLB, Promega Corp), diluted in distilled H_2O from 5 × stock solution. The 24-well-plate was placed on a plate shaker at 4°C for 15 min to ensure even coverage of all cells in each well. Complete lysis was ensured by a single freeze-thaw at 70°C for 40 min followed by thawing on a plate shaker at room temperature. The RLB was aspirated and placed in numbered Eppendorf microcentrifuge tubes. The tubes were centrifuged at 10000 g for 2 min at room temperature. 10 µl of cell lysate was added to a luminometer tube containing 50 µl of luciferase assay reagent (Promega Corp). As the luciferase assay reagent is light-sensitive only 12 samples were prepared at a time. The solutions were vortexed briefly before being measured by the luminometer.

2.5.4 β-Galactosidase assay

A constitutively active β-galactosidase control expression vector (PCH110) was co-transfected to control for transfection efficiency. Endogenous β-galactosidase was inactivated by incubating the cell lysate samples for 50 min at 48°C. The samples were centrifuged for 1 min at max speed. 10 µl of cell lysate was added to a luminometer tube containing 70 µl of reaction buffer (Galacton-plus substrate diluted 1 in 100 in reaction buffer dilutent; Tropix, Bedfort, MA, US), and incubated at room temperature for 1 h. 100 µl of light emission accelerator (Tropix) was added to each luminometer tube and each sample measured by the luminometer. To normalise the transfection each sample's luciferase reading was divided by the corresponding β-

galactosidase reading. This numerical reading was used as a comparison against other samples.

2.6 Protein analysis

2.6.1 Whole cell protein extraction

The supernatant was removed by aspiration and the cells placed on ice. The cells were washed gently by the addition of 4 ml/well of ice-cold PBS, which was subsequently removed. A volume of 250 µl/ 500,000 cells of lysis buffer (150 mM NaCl, 50 mM Tris; pH 7.4, 10 mM EDTA, 10 mM EGTA, 0.6 % NP40, 10 % glycerol, 10 µg/ml peptain and 1 mM PMSF) and protease inhibitor cocktail (protease inhibitor mini Tablets (Sigma); 1 Tablet dissolved in 10 ml of lysis buffer) was added to each well. The plate was rocked on ice for 15 min to ensure adequate lysing of all cells, after which the surface of the wells were scraped, with a dedicated cell scraper, and the lysate transferred into a fresh ice-cold Eppendorf microcentrifuge tube. The samples were centrifuged at 12000 g/ 15 min at 4°C. The resulting supernatant was aspirated into a fresh, ice-cold Eppendorf microcentrifuge tube. The sample was stored at -70°C until used.

2.6.2 Protein Assay

Total cell protein concentration was quantified using the DC Protein Microassay (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK). The protein assay is a dye-binding assay, based on the method of Bradford (Bradford 1976), in which a differential colour change of a dye occurs in response to various concentrations of protein. A control sample of distilled H_2O, bovine serum albumin (BSA) protein standards (0.125 mg/ml to 1.5 mg/ml) and the samples to be measured (25 µl/well) were aliquoted in duplicate to a clear 96-well Nunc-plate (Nunc, Invitrogen, Paisley, UK). Reagent A/S (25 µl/well) was added (1 ml Reagent A plus 20µl Reagent S followed by 100 µl/well of Reagent B (Bio-Rad Laboratories Ltd). The plate was incubated at room temperature for 15 min. The colour absorbance was measured at 690 nm in a Multiscan plate reader (Multiscan EX Labsystems). The

results were analysed by interpolation from the standard curve obtained from the standards of known concentrations.

2.6.3 Protein samples and electrophoresis

Protein samples (40 μg) were mixed with 5x SDS loading dye (50 mM Tris-HCl; pH 6.8, 100 mM DTT (dithiothreitol) 2 % SDS, 0.1 % bromophenol blue and 10 % glycerol with 0.01 % β-mercaptoethanol) and boiled for 3-4 min. The samples were loaded onto wells in a precast 4 % to 20 % gradient Tris-Glycine gel (Novex, Invitrogen) and immersed in 1x electrolyte running buffer (25 mM Tris, 250 mM glycine; pH 8.3 and 0.15 % SDS) in an X Cell Sure Lock electrophoresis tank (Invitrogen). 14 μl of a pre-stained SDS-page standard, SeeBlue™ (Invitrogen), was run in a parallel lane to the test samples to allow for identification of sample protein size. The gel was run at 100 volts for 90 min until the dye had run to the bottom of the gel.

2.6.4 Protein electrotransfer

Protein was electrotransferred onto polyvinylidene difluoride membranes (PVDF), which had been previously washed in methanol for 15 sec, followed by a wash in distilled water and finally a wash in transfer buffer (39 mM glycine, 48 mM Tris and 20 % methanol). The electrotransfer was performed on a Transblot® SD semi-dry transfer cell unit (Bio-Rad Laboratories, Ltd) set at 14 volts per membrane for 90 min. See Figure 2.2 for diagram of the electrotransfer.

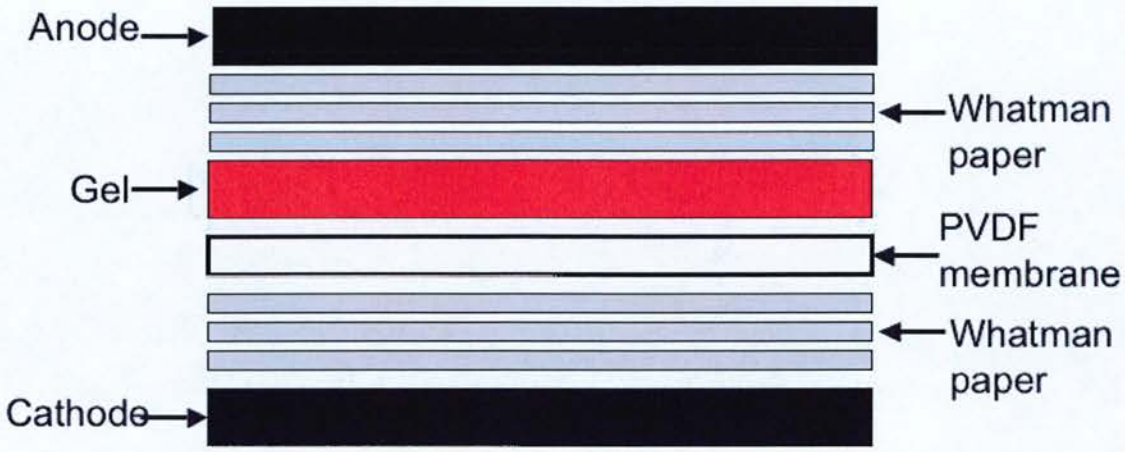


Figure 2.2: Electrotransfer apparatus

2.6.5 Western blot analysis

The electrotransferred membrane was removed form the unit and blocked for 1 h at 25°C with 15 ml of 4 % BSA + 4 % NaN_3. The primary antibody was added at the correct concentration diluted in 4 % BSA + 4 % NaN_3 for 18 h at 4°C. After washing three times with TNS Tween (50 mM Tris-HCl, 150 mM NaCl and 0.05 % Tween) for 10 min each at room temperature, membranes were subsequently incubated for 1 h with an HRP-conjugated secondary antibody directed against immunoglobulins of the host species in which the primary antibody was raised. After washing a further three times with TNS Tween for 10 min each at room temperature, blots were developed by chemiluminescence (ECL plus kit, Amersham Biosciences) for 20 min at room temperature, protected from light. The HRP reacted with the chemiluminescent substrate to create light emission at the site of antigen expression. The Enhanced Chemi-Luminescence reagents (ECL Plus™, Amersham Life Science, GE Healthcare, Buckinghamshire, UK) were prepared immediately prior to use, after equilibration to room temperature, in accordance with the manufacturer's advice. The chemiluminescence was detected using the typhoon 9400 phosphoimager (Amersham Biosciences, GE Healthcare).

2.6.6 Stripping and re-probe of membranes

Membrane-bound antibodies could be stripped away with stripping buffer (1.6 % SDS, 200 mM Glycine; pH 2.5) for 45 min at room temperature on a plate rocker. The membrane was washed and re-probed for β-Actin, which was used as an internal control to indicate relative loading of the samples.

2.6.7 Enzyme-linked Immunoabsorbant Assay (ELISA)

An enzyme-linked immunoabsorbent assay (ELISA) can be used to detect and measure various proteins released by cells during *in vitro* culture. The media in which the cells have been grown and treated was collected and stored at -20°C. The ELISA detects the amount of a particular protein present and measures this against a standard curve to give the concentration of protein present in the media sample. The assay is run on Maxisorp 96-well plates (Nunc). Method files for each ELISA were constructed in Assay Zap™ (AssayZap™, BioSoft, Cambridge, UK) and contained information concerning the range of standards used and the level of non-specific binding. This allowed a standard curve to be produced against which the samples were measured. In an ideal two site ELISA the response curve is a straight line, but deviation from the ideal (i.e. due to sub-optimal capture antibody concentration) will yield a sigmoid response curve. Results were analysed using a computer programme, AssayZap™, specifically designed for this purpose.

2.6.7.1 Two-site Sandwich ELISA

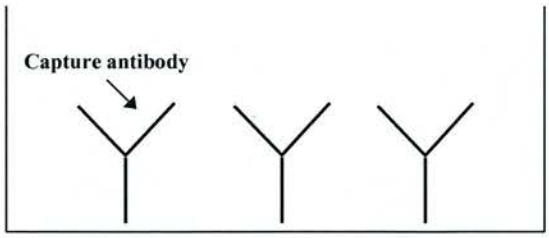
Prior to the assay, plates were passively coated overnight at 4°C with 100 µl/well of capture monoclonal antibody that was specific for the protein of interest (4 µg/ml). After incubation, plates were washed in water and 100 µl/well of blocking and protecting medium (2 % polyvinyl pyrollidone; 5 mg/ml BSA; 0.5 ml/litre preservatives (200 mg/ml 5-bromo-5-nitro-1,3-dione and 200 mg/ml 2-methyl-4-isothiazolin-3-one in DMF/DMSO 1:2); 5 mM EDTA; 50 mM EDTA) was added to prevent non-specific binding and reduce background recordings across all samples. After 30 min at room temperature the solution was discarded and plates were air

dried and stored at 4°C. Standards were diluted in ELISA buffer (1 L distilled H20; 100 mM Tris; 2 mg/ml BSA; 9 g NaCl; 2 mM EDTA; 300 µl phenol red solution; 1 ml preservatives, pH 7.2). The standards, quality controls (of a set concentration in multiple replicates), non-specific binding wells and finally the media samples to be assayed were incubated together (100 µl/well). Any free, specific protein in the wells spontaneously bound to the capture antibody on the base and sides of the well. Plates were sealed and incubated overnight at 4°C. Excess media was discarded and plates washed 4 times in wash buffer prior to addition of a biotinylated detection antibody (secondary) (50 ng/ml at 100 µl/well) the plate was incubated for 90 min on a plate shaker at room temperature, after which the plate was washed as aforementioned. This secondary antibody formed a complex with the previously bound protein and was detected with 100 µl/well of streptavidin peroxidase conjugate (SPC) (Roche, Hertfordshire, UK), diluted 1:2000 in assay buffer. The plate was incubated for 20 min on a plate shaker at room temperature. Unbound SPC was removed by washing. Subsequently, 200 µl/well peroxidase substrate, consisting of 20 ml sodium acetate (pH 6; 100 mM), 2 ml 0.5 % urea hydrogen peroxidase (pH 6) and 2 ml tetramethyl benzidine (3 mg/ml), was added. Any bound streptavidin peroxidase metabolised the hydrogen peroxide present in the substrate and the tetra methyl benzidine was converted into a coloured product. The reaction was stopped after 20 min incubation by addition of 50 µl/well 2 N sulphuric acid. The colour was then measured on a plate reader at 450nM. The details of the ELISA are summarised in Figure 2.3

Figure 2.3: Description of the stages and principles of a two-site sandwich ELISA.

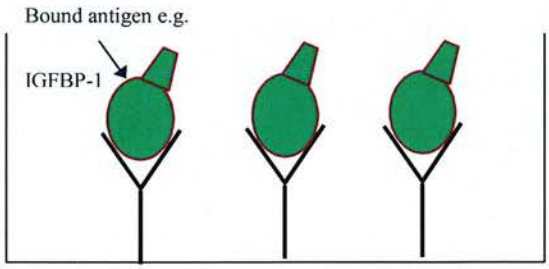
Stage 1:

The capture antibody is bound to the base and sides of the wells.



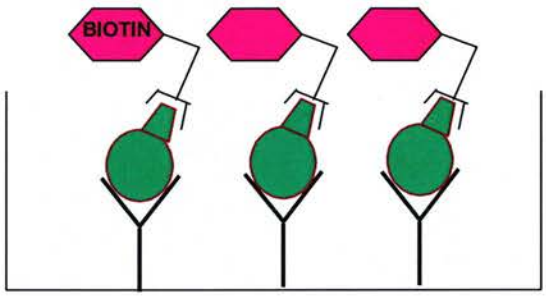
Stage 2:

Any antigens in the media specific to the capture antibody bind.



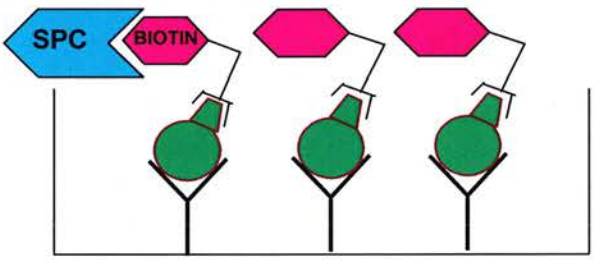
Stage 3:

A biotin-labelled detection antibody is added and it binds to the bound antigen.

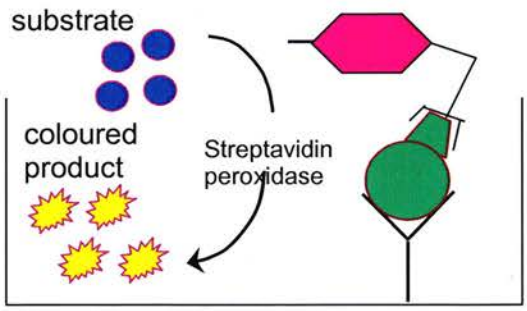


Stage 4:

Streptavidin peroxidase conjugate is added to the wells.



Stage 5: This converts the substrate to a coloured product that can be quantitatively measured.



2.6.8 Time-resolved fluoroimmunoassay of prolactin in culture supernatants

Culture supernatants were stored at -20 °C until assayed. Decidual prolactin (PRL) release was measured by a DELFIA® Prolactin time-resolved fluoroimmunoassay kit (PerkinElmer Life Sciences, Belgium). The fluoroimmunoassay is a solid phase, two-site assay based on the direct sandwich technique as described above. Briefly, 200 µl of anti-prolactin-EU tracer (see Figure 2.4), diluted 1:75, was added to the microtitration strips. Standards provided in the kit (of a set concentration in multiple replicates), and media samples to be assayed (all 25 µl/well) were added to the microtitration strips and incubated for 90 min at room temperature on a plate shaker. The standards and media samples reacted simultaneously with the immobilised mouse monoclonal antibody directed against a specific antigenic site on the PRL molecule and europium-labelled antibody directed against a different antigenic site. After incubation the microtitration strips were washed in the wash buffer supplied (Tris-HCL buffered (pH 7.8) salt solution with tween and Germall II as preservative) and enhancement solution (200 µl) was added to each well so as to avoid touching the sides of the wells. This enhancement solution dissociates europium ions from the labelled antibody into solution, where they form highly fluorescent chelates with components of the enhancement solution. The fluorescence of each sample is proportional to the concentration of PRL in the media sample and was measured on a time-resolved fluorometer, VICTOR™ 1420 Multilevel Counter (Wallac, PerkinElmer LAS (UK) Ltd, Beaconsfield, UK). The concentration of prolactin was determined by interpolation from a standard curve prepared from the prolactin standards. The assay was conducted according to the manufacturer's protocol.

Stage 1:

Solid phase anti-PRL IgG and Eu-labelled hPRL IgG bind to different antigenic sites on PRL.

Stage 2:

Enhancement solution is applied to the wells, dissociating europium ions from the Eu-labelled antibody.

Stage 3:

The dissociated Eu ions form fluorescent chelates. This fluorescence is measured by a time-resolved fluorometer and the PRL concentration determined by interpolation from a standard curve.

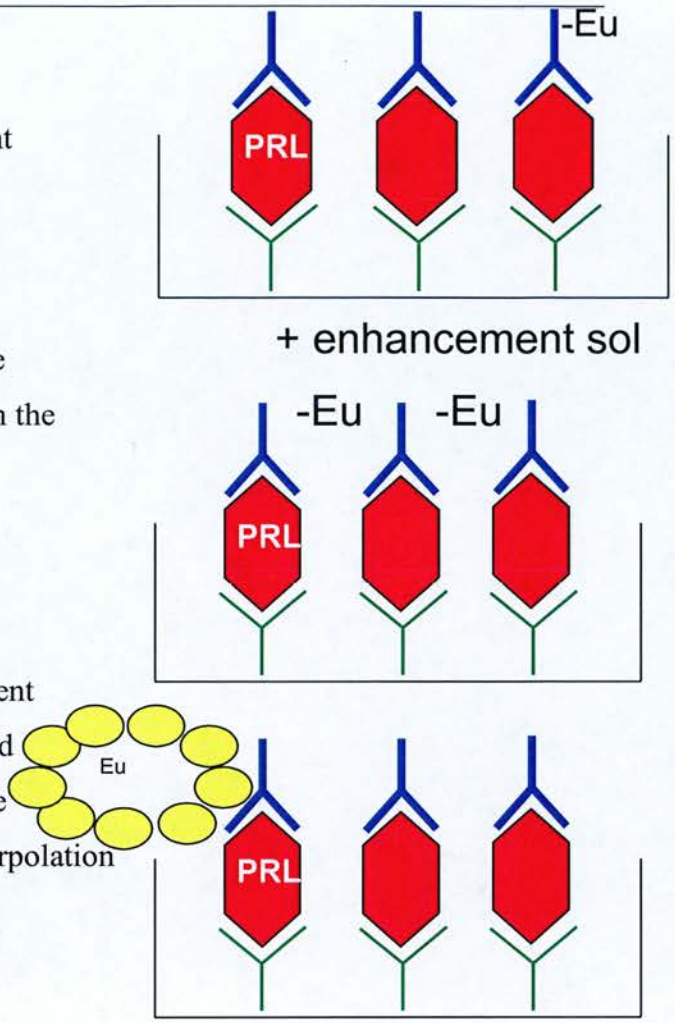


Figure 2.4: Description of the stages and principles of a solid phase, two-site fluoroimmunometric assay based on the direct sandwich tequnique.

2.6.9 Immunolocalisation of proteins and tissue sections

2.6.9.1 Preparation of slides

Tissue sections, embedded in paraffin wax blocks were cooled from room temperature prior to being cut. Cooling made the wax more rigid and easier to slice. The blocks were cut into 5 μm sections using a hand operated microtome RM 2135 (Leica Microsystems (UK) Ltd, Milton Keynes, UK). Sections were floated on distilled H_2O (42°C) and placed on charged slides. The slides were dried for at least 3 h at 50-60°C, usually overnight (Lamb RA, East Sussex, UK. model E28.5). Once

dry, the slides were dewaxed in xylene (Pioneer Research Chemicals Ltd) for 5 min then transferred into a second vial of xylene for a further 5 min. The sections were rehydrated in an alcohol series starting in absolute ethanol for 20 sec, and then into 95 % ethanol and finally 70 % ethanol, each for 20 sec. Slides were transferred into distilled H_2O and washed once in PBS. ESC, cultured on Labtec chamber well slides, were washed with cold PBS (Sigma), fixed in 4 % NBF for 2 h and washed with PBS.

2.6.9.2 Immunohistochemistry (IHC) and immunocytochemistry (ICC)

The IHC/ICC method used was a standard avidin-biotin peroxidase (ABC) method allowing specific proteins within fixed endometrial tissue/stromal cells to be detected and visualised. This method is based on the principal that avidin has a high binding affinity ($K = 10^{-15}M$) for biotin. The primary antibody will bind specifically to the protein of interest in the tissue section or cells. The biotinylated secondary antibody that was raised against specific immunoglobulins of the species in which the primary antibody was raised binds to antigenic sites on the primary antibody. Addition of an avidin-biotin and horse-radish peroxidase (HRP) enzyme complex resulted in any free sites on the avidin molecule binding to the biotin on the secondary antibody. HRP activity in the presence of an electron donor, such as 3, 3-diaminobenzidine (DAB), resulted in the formation of an enzyme-substrate complex and then oxidation of DAB. The resulting colour change of the chromogen, DAB, produced a brown precipitate, which was insoluble in alcohol and other organic solvents and was formed at the site of the protein of interest.

2.6.9.3 Antigen retrieval

Fixatives work by inducing intra-molecular cross-linkages. These can mask target proteins (antigens) and thus prevent their immunodetection. Antigen retrieval describes the process by which the cross-linkages are partially undone by the application of heat and change in pH. Treatment of the tissue sections to expose masked antigens involved pressure-cooking (Tefal) the slides in 0.01 M citrate buffer

(pH 6). Briefly, the slides were immersed in 2 L of boiling citrate buffer; the lid was sealed and set to 13 psi. The slides were heated until full pressure was reached and pressure cooked for 5 min. After which, the pressure was released and the pressure cooker removed from heat. The slides were left to stand in the buffer for 20 min. The sections were cooled and washed in distilled water followed by washing in PBS prior to the next stage of the imunohistochemical procedure. Treatment of the cell preparations to allow access to intracellular antigens involved permeabilisation of the cell membrane by incubating the slides for 20 min at room temperature with 0.2 % NP40 (Sigma), 1 % BSA (Sigma), and 10 % nonimmune goat serum (NGS; Diagnostics Scotland, Carluke, UK). Slides were washed with PBS.

2.6.9.4 Blocking endogenous peroxidase

Endogenous peroxidase activity was blocked with 3 % H_2O_2 in 60 % (v/v) methanol for 30 min at room temperature on a rocker. This step reduces background staining. The slides were subsequently rinsed in PBS or TBS before the next stage of the immunohistochemical procedure.

2.6.9.5 Normal serum block

Non-specific binding of components in the secondary antibody was minimised by the application of a dilute solution of serum from the species in which the secondary antibody was raised. For all IHC procedures goat serum, diluted 1:4 in TBS and BSA, was applied to the slides for 30 min at room temperature to block tissue due to be exposed to an anti–mouse secondary antibody raised in goats.

2.6.9.6 Avidin-biotin block

Endogenous biotin was blocked with the avidin-biotin blocking kit (Vector Laboratories Ltd, Peterborough, UK) to reduce non-specific staining. Avidin was added, neat, to the slides and incubated for 20 min at room temperature. The slides were washed three times in TBS for 5 min before biotin was added (neat), incubated at room temperature for 15 min followed by three washes in TBS for 5 min each.

2.6.9.7 Primary antibodies

The appropriate primary antibody (Table 2.5) was diluted as required in blocking serum/TBS/BSA, applied to the slides and incubated overnight at 4°C.

Antigen	Dilution	Antigen retrieval	Species raised in	Source
Cytokeratin	1:100	Permeabilise cell membrane/ Citrate	Mouse	DAKO
PR	1:40	Permeabilise cell membrane	Mouse	Novocastra
Vimentin	1:1000	Permeabilise cell membrane/ Citrate	Mouse	DAKO

Table 2.5: Details of primary antibodies for IHC

2.6.9.8 Secondary antibody

After incubation with the primary antibody the slides were washed three times with TBS for 5 min each, and incubated with the secondary antibody, goat anti-mouse biotinylated (Dako UK Ltd, Cambridgeshire, UK) at a dilution of 1:500 in the blocking serum (NGS/TBS/BSA) for 1 h at room temperature. The antibody was washed off with TBS as before.

2.6.9.9 Horse-radish peroxidase

The ABC-HRP detection system (Dako UK Ltd) was used to amplify the signal obtained from the biotinylated secondary antibody (Hsu, Raine et al. 1981). ABC-HRP was prepared by adding 1 drop of solution A (Dako UK Ltd) and 1 drop of solution B (Dako UK Ltd), approximately 20 µl each, to 5 ml of Tris-HCl. This was

made up 30 min prior to application. The ABC-HRP was added to the slides for 30 min at room temperature followed by three washes in TBS for 5 min each.

The ABC-HRP complex works on the principal that avidin forms a tertiary structure and possesses four binding sites for biotin. However, due to the molecular orientation of the binding sites, fewer than four molecules of avidin are occupied with biotin molecules complexed to HRP, leaving at least one site empty for binding to the biotinylated secondary antibody (Figure 2.5).

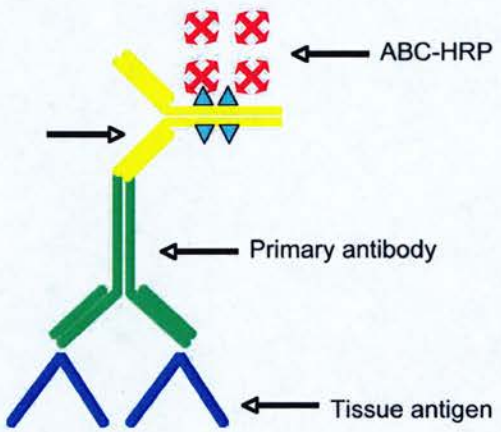


Figure 2.5: ABC-HRP detection system (adapted from Dako UK Ltd. Handbook of Immunochemical staining methods 3rd Edition, Boenisch et al, 2001).

2.6.9.10 Developing, counterstaining and mounting

The slides were developed and antigenic sites were visualised using DAB; 1 drop per ml in DAB buffer (Dako UK Ltd). After DAB staining, the slides were counterstained with Harris' haematoxylin (Pioneer Research Chemicals Ltd., Colchester, UK), differentiated in acid-alcohol and the nuclei developed in Scott's tap water (Pioneer Research Chemicals Ltd). The slides were dehydrated in increasing concentrations of alcohol and cleared in histoclear (Pioneer Research Chemicals Ltd) and xylene for 5 min each before mounting under a borosilicate glass coverslip (VWR) using Pertex™ (Cellpath plc, Hemel Hempstead, UK), a solvent based glue.

2.6.9.11 Photomicroscopy

Tissue sections and cell preparations were examined and images captured using an Olympus Provis microscope (Olympus Optical Co. London, UK) and a Kodak DCs330 camera (Eastman Kodak Co. Hemel Hempstead, Hertfordshire, UK) respectively. Images were compiled using the computer program, Photoshop CS (Adobe Systems, California, USA).

2.6.10 Immunofluorescence

Immunofluorescence is the localisation of antigens in tissue sections or cell preparations by the use of labelled antibodies as specific reagents through antigen-antibody interactions that are visualised by a fluorescent dye marker. Fluorescence results when sensitive materials are irradiated by light at a specific wavelength. This excites atoms within the compound to a higher energy level so that when they drop back down, the energy they release is detected as visible light. This emitted light has a longer wavelength and lower energy than the "exciting" light, generated by a laser beam incorporated into the microscope. To distinguish between the two types of light, the microscope is fitted with two filters, one of which enables the sample to be irradiated by light at a specific wavelength, the second separates out the excitation light from the emitted light. The colour of emitted light seen depends on its wavelength which varies depending on the sensitive chemical used. The fluorescent chromagens used are summarised in Table 2.6.

2.6.10.1 Antigen retrival

The tissue sections and cell preparations were treated as described in section 2.6.9.3.

2.6.10.2 Blocking endogenous peroxidase

Endogenous peroxidase activity was blocked as described in section 2.6.9.4. This prevents auto fluorescence.

2.6.10.3 Normal serum block

Non-specific binding was blocked as described in section 2.6.9.5, the only exception being that the serum was diluted 1:4 in PBS and BSA.

2.6.10.4 Avidin-biotin block

Endogenous biotin was blocked as described in section 2.6.9.6.

2.6.10.5 Primary antibodies

The appropriate primary antibody (Table 2.6) was diluted as required in blocking serum/PBS/BSA, applied to the slides and incubated overnight at 4°C.

Antigen	Dilution	Antigen retrieval	Species raised in	Source
CD56	1:250	Citrate	Mouse	Zymed
MMR1	1:10	Permeabilise cell membrane	Mouse	Abcam
hCG	1:20	Permeabilise cell membrane/ Citrate	Rabbit	Abcam
p-SMAD-2	1:100	Permeabilise cell membrane	Mouse	Abcam

Table 2.6: Details of primary antibodies for IHF

2.6.10.6 Secondary antibody

After incubation with the primary antibody the slides were washed once with PBS and 0.01 % Tween (PBST) for 5 min followed by two washes with PBS for 5 min each. The slides were incubated with the appropriate secondary antibody, at a

diltution of 1:500 in PBS for 30 min at room temperature. The antibody was washed off with PBST and PBS as before.

Antibody label	Dilution	Species raised in	Source
Biotinylated	1:500	Goat	DAKO
Peroxidase	1:500	Goat	DAKO

Table 2.7: Details of secondary antibodies for IHF

2.6.10.7 Streptavidin Alexaflour 488/546

The slides, which were previously incubated with a biotinylated-labelled secondary antibody, were incubated in Streptavidin alexaflour 488/546 (Molecular Probes), diluted 1:200 in PBS, for 1 h at room temperature, in a light protective humidified chamber. The streptavidin alexaflour binds in a similar manner to the ABC-HRP detection system described in section 2.6.9.9. After incubation with streptvidin alexaflour 488/546 the avidin was washed off with PBST and PBS as described previously and protected from light.

2.6.10.8 Tyramide detection

The slides, which were previously incubated with a peroxidase-labelled secondary antibody, were incubated for 10 min with tyramide Cy3 (TSA plus cyanine 3 system; Perkin-Elmer Life Sciences, Boston, MA) diluted 1:50 in the buffer supplied, to amplify immunostaining and produce red fluorescence.

Fluorescent complex (Supplier)	Dilution	Exposure duration/	Excitation wavelength (nm)/ emitted light colour
Tyramide Cy3 (Perkin-Elmer Life Sciences)	1:50	10 min	546/ red
Streptavidin conjugated alexa 488 (Molecular Probes)	1:200	1 h	488/ green
Streptavidin conjugated alexa 546 (Molecular Probes)	1:200	1 h	546/ red
To-Pro 3 (Molecular Probes)	1:2000	2 min	655/ blue
DAPI (Molecular Probes)	1:1000	10 min	405/ turquoise

Table 2.8: Details of fluorescent labelled complexes used.

2.6.10.9 Developing, counterstaining and mounting

The cells were counterstained with To-Pro-3 (Molecular Probes, Invitrogen), diluted 1:2000 for 2 min, or DAPI (Molecular Probes, Invitrogen), diluted 1:1000 for 10 min, in a light protective humidified chamber, to visualise the cell nuclei. The slides were washed as described previously for a final time and mounted under a glass coverslip using Permafluor™ mounting medium (Beckman Coulter, High Wycombe, UK), a water based glue.

2.6.10.10 Confocal imaging

Slides immunostained with a fluorescent antibody were observed on a LSM 510 laser scanning confocal microscopy (Zeiss, Hertfordshire, UK) using the 405 nm laser to

visualise turquoise fluorescence, Argon 488 nm laser to visualise green fluorescence, the HeNe1 546 nm laser to visualise red fluorescence, and the HeNe2 633 nm laser to visualise blue fluorescence. The images were compiled on the computer programme, Photoshop CS.

2.6.10.11 Image analysis

Images captured on the confocal were analysed on the program, Image Pro Plus (Media Cybernetics, USA). This program measures the mean optical density of the fluorescence in the cell nucleus and allows comparison with other images when captured at identical settings.

2.7 Fibroblast Cell Purity

Cultures of ESCs were grown for two weeks before the purity of fibroblasts in the cultures (n = 6) was determined both by Fluorescence Activated Cell Sorting (FACS) (Beckman Coulter) and IHC.

2.7.1 FACS

Cells were labelled using an antibody specific for a surface antigen found on human fibroblasts (CD90, Oncogene, Dako UK Ltd). Approximately 1.5 million ESCs were removed from culture following trypsinisation, re-suspended in 300 µl FACS buffer (PBS, containing 0.1 % N_aN_3 and 1 % FCS) and split between three 1.5 ml Eppendorf tubes: 10 µl of the anti-CD90 antibody was added to the first tube, 10 µl anti-mouse IgG whole molecule with FITC conjugated (Sigma) was added to the second tube and the third tube remained untreated. The cells and solutions within the Eppendorfs were mixed and all three tubes were left on ice for 30 min in the dark. All three tubes were centrifuged at 1500 g for 1 min to pellet the cells which were re-suspended in 1 ml FACS buffer. This wash was repeated twice. To all tubes 100 µl FACS buffer was added and cells re-suspended, tubes were wrapped in foil to prevent light exposure. Before sorting the cells in a Beckman Coulter® FACS caliber® Flow Cytometer the suspensions were passed though cell strainers (Becton

Dickinson Labware, Europe, France) to ensure a single cell suspension. The cells were passed through an aperture as single cells. The laser beam detected cells and registered them as an event. The laser beam determined two properties of each cell: the granularity and cell size. The granularity was read by the amount of side scatter of the laser beam and the cell size determined by the degree of forward scatter. The third measurement made by the laser beam was fluorescence, in this case fluorescein. The three measurements combined illustrated the distinct populations of cells that were present and the percentage of fluorescence provided the percentage of fibroblast-positive cells in the sample. The purity of the fibroblasts is shown in Figure 2.6.

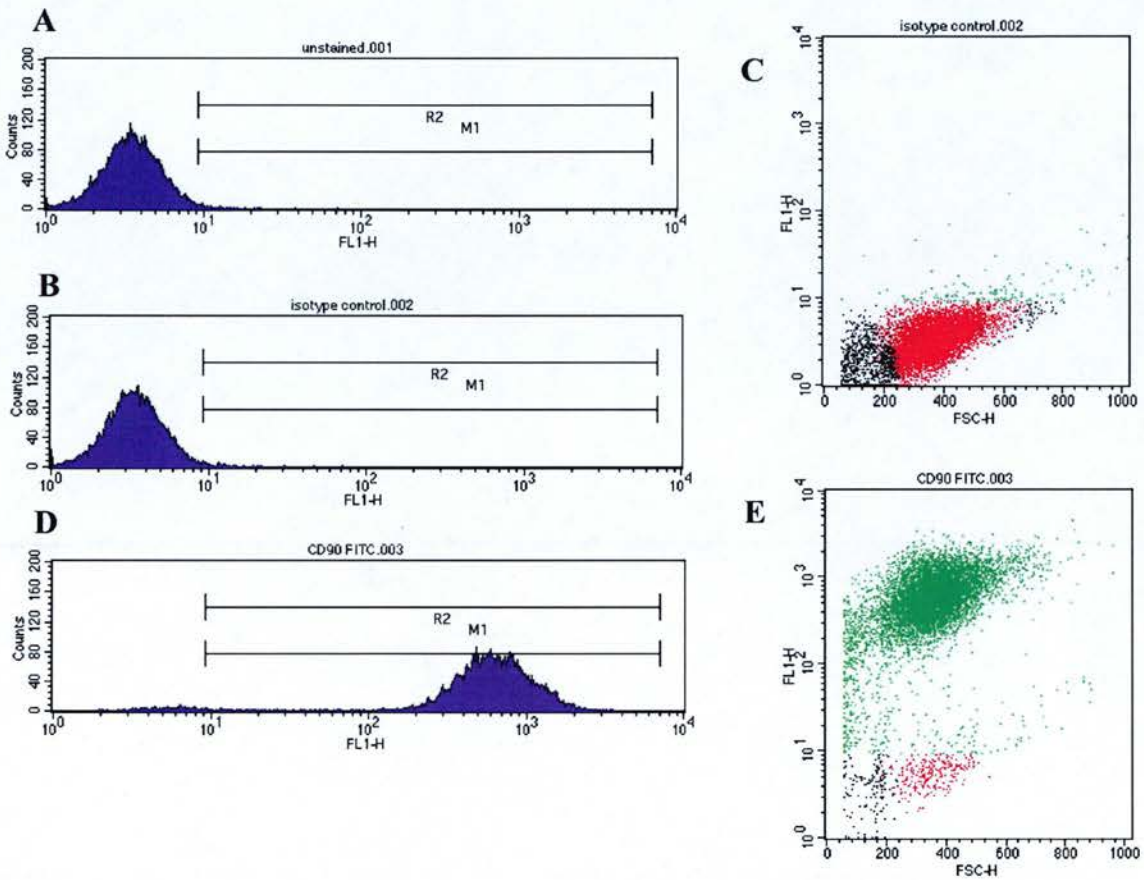


Figure 2.6: FACS analysis of ESC cell purity. Panel A shows unstained cells, panel B shows isotype FITC control, panel C shows the forward and side scatter plot for isotype control cells, red depicts cells without and green depicts cells with florescence, panel D shows CD90 FITC stained cells, panel E shows the forward and side scatter plot for CD90 FITC-stained cells, green depicts cells with florescence. Representative scans from 35 samples are shown; cells are typically > 96 % pure.

2.7.2 IHC detection of fibroblast purity

Cells were seeded in Labtec culture slides at a concentration of 500,000 cells per well and immunostained using antibodies directed against vimentin, a cytoplasmic fibroblast cell marker, or cytokeratin, an epithelial cell marker, as follows.

Cultured cells were washed with cold PBS (Sigma) and then fixed in 4 % NBF for 2 h. Thereafter, cells were washed with PBS and permeabilised by incubating the slides for 20 min at room temperature with 0.2 % NP40 (Sigma), 1 % BSA (Sigma), and 10 % nonimmune goat serum (NGS; Diagnostics Scotland). Non-specific binding of primary antibody was blocked by incubating slides in a 1:5 dilution of NGS in TBS (Tris-buffered Saline) containing 5 % BSA (NGS/TBS/BSA). Endogenous avidin/biotin was blocked using a commercially available avidin-biotin blocking kit (Vector Laboratories Ltd) then washed twice with PBS. Slides were incubated at 4°C overnight in a 1:40 dilution of mouse monoclonal anti-human vimentin (Dako UK Ltd) or a 1:40 dilution of mouse monoclonal anti-human cytokeratin (Dako UK Ltd) made up in NGS/TBS/BSA. Antibody binding was detected by applying a 1:500 dilution of biotinylated goat antimouse antibody (Dako UK Ltd) in NGS/TBS/BSA, followed by an avidin/biotin horseradish peroxidase complex (Dako UK Ltd), for 60 min and 30 min respectively, at room temperature. Finally, antigenic sites were visualised by DAB (Dako UK Ltd) before counterstaining in Harris's hematoxylin (Pioneer Research Chemicals Ltd), dehydrating, and mounting with Pertex mountant (Cellpath plc). Fibroblast purity is shown in Figure 2.7.

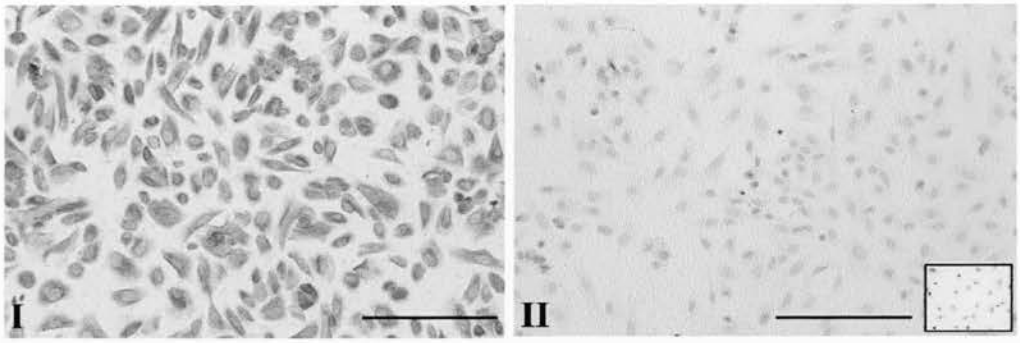


Figure 2.7: Fibroblast purity demonstrated by IHC. Panel I shows ESCs stained for vimentin, a fibroblast marker, and panel II shows ESCs stained for cytokeratin, an epithelial cell marker. Insert shows negative control. Representative slides are shown. Scale bars represent 100 μm.

2.8 Statistical Analysis

Prior to any statistical analysis data was tested for and shown to exhibit Gaussian distribution. Gaussian distribution was determined by applying the Shapiro-Wilk normality test to the data. Where appropriate, values were presented as means ± S.E.M. Comparison of the different parameters for the various treatment groups was determined by repeated measures analysis of variance (ANOVA). Significant differences were assigned using Kruskal-Wallis post hoc test. The criterion for significance for all tests was set at $p < 0.05$. Specific software was used to assist in the data analysis (GraphPad Prism v4.0b for Macintosh, GraphPad Software, San Diego, USA).

Chapter 3

3 Impact of TGFβ on gene expression in non-decidualised and decidualised stromal cells

3.1 Introduction

3.1.1 Progesterone receptor

In the normal menstrual cycle, increasing concentrations of oestrogens, produced by the ovaries, induce proliferation of endometrial glandular, stromal and vascular endothelial cells, producing a notable thickening of the endometrium, across the proliferative phase. Progesterone requires the presence of its nuclear receptor to induce differentiation of the endometrium and progesterone receptor (PR) expression requires prior exposure to oestrogen. Indeed, the presence of PR is considered evidence of a functional ER-mediated pathway. In mice, functional PR is crucial to female fertility with PR A/ PR B double knockouts displaying pleiotropic reproductive abnormalities (Lydon, DeMayo et al. 1995). PR B -/- mice do not have any impairment of ovarian or uterine function, but PR A -/- mice have severely impaired ovulation and lack decidualisation of the endometrial stromal cells (Conneely, Mulac-Jericevic et al. 2001).

In the human, expression of PR varies temporally and spatially, in both functional and basal regions and within the epithelial and stromal compartments, across the normal menstrual cycle (Garcia, Bouchard et al. 1988; Lessey, Killam et al. 1988; Critchley, Bailey et al. 1993; Snijders, de Goeij et al. 1996; Wang, Critchley et al. 1998).

3.1.2 Transforming Growth Factor β 1

Transforming Growth Factor β 1 (TGFβ1), a secreted homodimeric protein, is the prototypic member of a family of approximately 40 structurally related proteins known as the "TGFβ superfamily". TGFβ1 and its isoforms regulate a plethora of diverse biological functions (Ignotz and Massague 1986; Desmouliere, Geinoz et al. 1993; Fajardo, Prionas et al. 1996; Jester, Barry-Lane et al. 1996; Sankar, Mahooti-Brooks et al. 1996; Sandberg, Casslen et al. 1998; Serini, Bochaton-Piallat et al. 1998; Lewis, Lygoe et al. 2004; Shephard, Martin et al. 2004). TGFβ1 has been shown to enhance tissue remodelling and homeostasis in endometrial cells (Bruner, Rodgers et al. 1995; Ulloa, Creemers et al. 2001) and inactivation of TGFβ1 has been implicated in endometrial carcinogenesis (Parekh, Gama et al. 2002).

TGFβ1 is present in its latent form in the endometrium until the late secretory phase when it is activated by plasmin (Lyons, Gentry et al. 1990). Plasmin is formed from inactive plasminogen by urokinase plasminogen activator (uPA), which is itself regulated by plasminogen activator inhibitor (PAI-1) (Loskutoff, Sawdey et al. 1993), as detailed in section 1.5.1.12.1. A previous study reported that TGFβ1 stimulates the synthesis of PAI-1, demonstrating a negative feedback loop on its own production. In addition, progesterone has been shown to regulate the release of PAI-1 in stromal cells *in vitro* (Casslen, Andersson et al. 1986). Furthermore uPA is expressed in the late secretory phase in co-ordinance with falling progesterone levels prior to menstruation (Casslen, Sandberg et al. 1998), possibly indicating the existence of a relationship between progesterone and TGFβ1 (Sandberg, Eriksson et al. 1997).

3.1.3 Transforming Growth Factor Beta 1 signalling

TGFβ1 initiates its diverse cellular responses by stimulating formation of specific heteromeric complexes of type I (ALK 5) and type II serine/threonine kinase transmembrane receptors located at the cell surface. The type II receptor phosphorylates type I in the juxtamembrane region (GS domain, rich in glycine and serine residues), which in turn propagates the signal intracellularly via the phosphorylation of highly conserved members of receptor-regulated SMAD (Sma- and mothers against decapentaplegic (MAD) -related protein) family of transcriptional regulators, SMAD 2 and 3 (Graff, Bansal et al. 1996; Macias-Silva, Abdollah et al. 1996; Nakao, Imamura et al. 1997; Massague and Wotton 2000; Shi and Massague 2003), as previously detailed in section 1.6.1.

3.1.4 Protein inhibitor of activated STATs

Protein inhibitor of activated STAT (signal transducer and activator of transcription) (PIAS) represents a family of proteins originally identified through interaction with cytokine-induced STAT (Chung, Liao et al. 1997). In mammals, five PIAS proteins have been identified, PIAS 1,3, xα, xβ and γ (Liu, Liao et al. 1998). PIAS 1 and 3 bind and inhibit STAT 1 and STAT 3 DNA-binding activities, respectively (Chung, Liao et al. 1997; Liu, Liao et al. 1998). PIAS 1, PIAS 3, PIAS xα and PIAS xβ are also reported to mediate steroid receptor-dependent transcriptional activation (Kotaja, Aittomaki et al. 2000). Moreover, the PIAS family has been shown to possess sumoylation ligase activity, which can promote the covalent addition of a small ubiquitin-related modifier to target proteins (Johnson and Gupta 2001; Sachdev, Bruhn et al. 2001; Nishida and Yasuda 2002; Lin, Liang et al. 2003). Sumoylation, the addition of the small ubiquitin-related modifier (SUMO), is a post-translational modification of proteins and can be involved in various cellular processes, such as nuclear-cytosolic transport, transcriptional regulation, apoptosis, protein stability, response to stress, and progression through the cell cycle (Verger, Perdomo et al. 2003).

PIAS γ is reported to inhibit STAT 1 mediated transcriptional responses without blocking their DNA-binding activities (Gross, Liu et al. 2001; Liu, Gross et al. 2001) and antagonises Wnt-independent and Wnt-induced transcriptional activation of lymphoid enhancer factor 1 (LEF 1) (Sachdev, Bruhn et al. 2001). Furthermore, PIAS γ has been reported to greatly augment the sumoylation of LEF 1 in an *in vitro* system, indicating that PIAS γ functions as a small ubiquitin-like modifier protein (SUMO) E3 ligase (Sachdev, Bruhn et al. 2001). A possible explanation for the repression of LEF 1 activity is facilitated via PIAS γ-mediated sequestration of LEF 1 to subnuclear structures that colocalise with SUMO (Sachdev, Bruhn et al. 2001). TGFβ1 induces expression of endogenous PIAS γ and in turn, PIAS γ interacts with SMAD 3 and antagonises SMAD 3-dependent transcriptional activation by TGFβ type 1 receptor, thereby providing a negative feedback mechanism for regulation of TGFβ1 signalling (Imoto, Sugiyama et al. 2003). Recently, Zoumpoulidou *et al* (Zoumpoulidou, Jones et al. 2004) reported that PIAS γ is complexed to PR and its ability to inhibit STAT signalling is dependent upon activation of PR. In human endometrium, cAMP-activated PKA induces the expression and activation of several transcription factors including STAT 5 (Mak, Brosens et al. 2002) capable of binding to PR.

3.1.5 Transforming growth factor β 1 and Wnt signalling cross-talk

Recent studies have shown that synergistic activation of Xtwn, a Wnt and TGFß target gene, is mediated by a physical association between intracellular components of these two pathways, namely, SMAD 3 and LEF1/TCFs (Letamendia, Labbe et al. 2001). The lymphoid enhancer binding factor/T-cell-specific factors (LEF1/TCF) are a family of DNA-binding proteins that associate with ß-catenin and thereby the activation of Wnt pathway gene targets. In addition, it has been reported that in response to TGFβ1 binding to its receptors, and activating the SMAD signalling pathway, expression of LEF 1 is induced (Medici, Hay et al. 2006). In Xenopus, β-catenin and LEF1/TCF form a complex with SMAD 4 (Nishita, Hashimoto et al. 2000).

It has been shown that Wnt-signalling is inhibited by the secreted protein DKK. DKK has been demonstrated to inhibit Wnt-signalling by binding to a low-density lipoprotein receptor-related protein, LRP 6 and inhibits signalling by disrupting the binding of LRP6 to the Wnt/Fz ligand-receptor complex (Wodarz and Nusse 1998; Tamai, Semenov et al. 2000; Mao, Wang et al. 2001). It has been reported that DKK mRNA expression is significantly upregulated in the stromal cells in the secretory phase of the cycle, suggesting that progesterone stimulates DKK expression and implying a role for DKK in decidualisation of the endometrium (Tulac, Nayak et al. 2003).

3.1.6 Hypothesis

Decidualisation is governed by progesterone acting via its nuclear receptor (Huang, Tseng et al. 1987; Zhu, Huang et al. 1990; Tseng, Gao et al. 1992; Mizuno, Tanaka et al. 1998; Brosens, Hayashi et al. 1999), which is only retained in the stroma during the late-secretory phase of the cycle (Wang, Critchley et al. 1998). Recently, the Wnt-signalling pathway antagonist, DKK, has also been implicated in the process of decidualisation ((Tulac, Nayak et al. 2003; Giudice 2004); (Tulac, Overgaard et al. 2006). In co-ordinance with decidualisation, TGFβ1 is active and abundantly expressed in both endometrial epithelial and stromal cells, and is proposed to play a critical role in cyclic tissue remodelling and inflammatory events associated with menstruation (Bruner, Rodgers et al. 1995; Ulloa, Creemers et al. 2001). However whether TGFβ1 affects either PR or DKK to regulate the decidual process is as yet unknown. We hypothesise that TGFβ1 may interact with PR and/or DKK to modulate the mechanism of decidualisation.

3.1.7 AIMS

The studies described in this chapter were designed to determine the effect of TGFβ on gene expression in non-decidualised and decidualised stromal cells. All studies utilised primary endometrial stromal cells isolated from non-pregnant endometrium as described in section 2.1.1. Some experiments included T47D cells, which were

utilised to identify if TGFβ1-induced responses were stromal cell specific. Experiments in this chapter were designed to address the following questions:

1: Do non-decidualised and decidualised ESCs possess TGFβ receptors, essential to facilitate TGFβ-induced responses (Massague and Wotton 2000)?

2: As previous studies suggest the existence of a possible relationship between TGFβ and progesterone (Sandberg, Eriksson et al. 1997), which isoform of TGFβ is most potent at inducing a change in PR expression?

3: Previous studies have also identified TGFβ1 as a potent inducer of a myofibroblast phenotype (Desmouliere, Geinoz et al. 1993; Jester, Barry-Lane et al. 1996; Serini, Bochaton-Piallat et al. 1998; Lewis, Lygoe et al. 2004; Shephard, Martin et al. 2004). To ensure that TGFβ1 is acting in the expected manner does TGFβ1 upregulate expression of SMAα in non-decidualised and decidualised ESC, and does a correlation exist between endogenous TGFβ1 expression and SMAα expression?

4: In the normal menstrual cycle PR A + B is upregulated in response to ovarian oestradiol. Does oestradiol upregulate PR A + B in the culture system used in this chapter and does TGFβ1 negate this response? Furthermore, does TGFβ1 downregulate PR A + B in decidualised ESCs and T47D cells?

5: Recent studies have identified a convergence between the Wnt-signalling pathway and TGFβ-signalling (Letamendia, Labbe et al. 2001). Does TGFβ1 interact with the Wnt-signalling antagonist DKK?

6: Does TGFβ1-mediated signalling have an impact on hormone-response elements of the steroid receptors (PR, ER, AR and GR) to inhibit the transactivation potential of the receptor and hence provide a potential mechanism for TGFβ1-induced responses?

7: Does TGFβ1 signal via the SMAD signalling pathway in the culture system used in this chapter, and does selectively silencing SMAD 4 prevent any of the observed TGFβ1-induced responses?

3.2 Methods

3.2.1 Human uterine tissue collection

Endometrial (n = 35) samples were collected as detailed in section 2.1.1 (Table 2.1a). Biopsies utilised in this chapter are detailed in Table 3.1. All endometrial samples were processed to isolate the stromal compartment from the glands, as described in section 2.2. Endometrial biopsies were dated according to the criteria of Noyes *et al* (Noyes, Hertig et al. 1950) and were found to be consistent with the patients' reported last menstrual period. In addition, all subjects had a serum sample collected at the time of biopsy for the determination of circulating oestradiol and progesterone levels by RIA as described by Yong et al (Yong, Glasier et al. 1992). Biopsies were classified as early, mid or late proliferative, or early, mid or late secretory, and a significant reduction in circulating progesterone levels was evident between biopsies collected in the mid and late secretory phases.

Patient number	Age	Day of Cycle	Blood E_2 (pg/ml)	Blood P (ng/ml)	Ethics No
1	49	20	608	34.9	17
2	51	20	75	2.1	17
3	37	6	215	1.8	17
4	33	11	373	1.8	17
5	39	13	964	3.9	17
6	37	7	234	1.7	17
7	33	6	255	2.8	17
8	44	13	359	1.8	17
9	41	31	650	51	17
10	44	25	276	42.1	17
11	39	10	835	10.14	17
12	42	14	478	23.66	17
13	41	9	1002	13.68	17
14	42	18	401	72.28	17
15	37	23	745	127.2	17
16	48	20	552	78.9	17
17	37	10	N/A	N/A	17
18	38	10	353	63	17
19	21	16	589	20.82	17
20	35	~ 27	398	63	17
21	47	11	1796	2.32	17
22	46	16	574	4.44	40
23	30	4	242	6.15	40
24	43	8	581	68.94	17
25	32	12	1651	4.04	17
26	33	22	520	62.68	17
27	40	12	667.03	10.86	17
28	43	16	607.2	146.41	17
29	42	8	208.52	0.88	17
30	39	22	516.89	93.23	73
31	46	15	641.34	2.5	17
32	35	9	278.52	2.45	17
33	47	26	113.18	60.4	17
34	30	~ 27	166.18	7.61	17
35	45	~ 27	323.11	27.3	17

Table 3.1:

Details of biopsies used in the studies presented in Chapter 3.

Stage of Cycle	Number of Biopsies
Menstrual	0
Early Proliferative	1
Mid Proliferative	12
Late Proliferative	6
Early Secretory	4
Mid Secretory	2
Late Secretory	6

Table 3.2: Summary of biopsy cycle stage from samples used in the studies presented in Chapter 3. Biopsies were staged according to the criteria of Noyes *et al*, with consistent E_2 and P levels at time of biopsy.

3.2.2 *In vitro* primary cell culture experiments

ESCs and T47D cells were maintained at 37°C in 5 % (v/v) CO_2 in RPMI 1640 medium (Sigma) supplemented with 10% FCS (Mycoplex), penicillin (50μg/ml; Sigma), streptomycin (50μg/ml; Sigma) and gentamycin (5μg/ml; Sigma) in the presence of ITS (Sigma) for T47D cell culture (section 2.2). Passaging of the cells was performed every 3-4 days by trypsinisation (section 2.2). The cells were seeded in 6-well plates at a concentration of 2.5 x 10^5 cells/ml and allowed to adhere and attain 90% confluence: 24 h prior to treatment the media was changed to serum-free RPMI. Tables 3.3-3.9 detail the treatment regimes utilised. Each treatment was duplicated and each experiment was repeated 3-9 times with separate endometrial biopsies.

Experiment 3.1: Are TGFβ receptors present in non-decidualised and decidualised stromal cells?		
Treatment	Concentration	Incubation period
Control ESC	N/A	72 h
MPA+8-Br-cAMP	1μM +250 μM	72 h

Table 3.3: Experimental regime for experiment 3.1

Experiment 3.2: Which isoform of TGFβ is most potent?		
Treatment	Concentration	Incubation period
Control	N/A	72 h
TGFβ1	10 ng/ml	72 h
TGFβ2	10 ng/ml	72 h
TGFβ3	10 ng/ml	72 h

Table 3.4: Experimental regime for experiment 3.2

Experiment 3.3 Does TGFβ1 interfere with the expression of SMA in non-decidualised and decidualised cells.		
Treatment	Concentration	Incubation period
Control	N/A	2, 12, 24, 36, 48, 72 h
MPA +8-Br-cAMP	1 μM + 250 μM	2, 12, 24, 36, 48, 72 h
TGFβ1	10 ng/ml	2, 12, 24, 36, 48, 72 h
MPA+8-Br-cAMP +TGFβ1	1 μM + 250 μM +10 ng/ml	2, 12, 24, 36, 48, 72 h

Table 3.5: Experimental regime for experiment 3.3

Experiment 3.4 Does TGFβ1 inhibit upregulation of PR in non-decidualised ESC in response to Oestradiol treatment?		
Treatment	Concentration	Incubation period
Control	N/A	2, 12, 24, 36, 48, 72 h
Oestradiol	0.1 μM	2, 12, 24, 36, 48, 72 h
TGFβ1	10 ng/ml	2, 12, 24, 36, 48, 72 h
Oestradiol +TGFβ1	0.1 μM +10 ng/ml	2, 12, 24, 36, 48, 72 h

Table 3.6: Experimental regime for experiment 3.4

Experiment 3.5: Does TGFβ1 interfere with the expression of PR in non-decidualised, decidualised cells and T47D cells.		
Treatment	Concentration	Incubation period
Control	N/A	2, 12, 24, 36, 48, 72 h
MPA +8-Br-cAMP	1 μM + 250 μM	2, 12, 24, 36, 48, 72 h
MPA+8-Br-cAMP +TGFβ1	1 μM + 250 μM +10 ng/ml	2, 12, 24, 36, 48, 72 h

Table 3.7: Experimental regime for experiment 3.5

Experiment 3.6: Does TGFβ1 interfere with the expression of DKK in non-decidualised, decidualised cells.		
Treatment	Concentration	Incubation period
Control	N/A	2, 12, 24, 36, 48, 72 h
MPA +8-Br-cAMP	1 μM + 250 μM	2, 12, 24, 36, 48, 72 h
TGFβ1	10 ng/ml	2, 12, 24, 36, 48, 72 h
MPA+8-Br-cAMP +TGFβ1	1 μM + 250 μM +10 ng/ml	2, 12, 24, 36, 48, 72 h

Table 3.8: Experimental regime for experiment 3.6

Experiment 3.7: Does neutralising endogenous TGFβ1 abrogate TGFβ1-induced responses.		
Treatment	Concentration	Incubation period
Control	N/A	72 h
MPA +8-Br-cAMP	1 μM + 250 μM	72 h
MPA+8-Br-cAMP +TGFβ1	1 μM + 250 μM +10 ng/ml	72 h
MPA+8-Br-cAMP + Anti-TGFβ1	1 μM + 250 μM + 2 ng/ml	72 h
Mouse IgG1		72 h

Table 3.9: Experimental regime for experiment 3.7

3.2.3 RT-PCR

Total RNA was extracted from the ESC and T47D cells as described in section 2.3.1 and cDNA prepared from the experiments detailed in section 2.3.2.

3.2.4 Semi-Quantitative PCR

Primers were designed to amplify the TGFβ1 receptor type 1 (TGFβ1R1) gene. PCR was carried out using BioMix Red (Bioline). Conditions for each sample were as follows:

2 x Bioline Biomix Red	10μl
TGFβ1R1 sense primer (0.5 μM)	2 μl
TGFβ1R1 antisense primer (0.5 μM)	2 μl
RNAse-free H_2O	2 μl
cDNA	4 μl

30 cycles of amplification were performed with an initial denaturing temperature of 95°C for 5 min for 1 cycle followed by 30 cycles of: denaturation at 95°C for 30 sec; specific TGFβ1 receptor type 1 primer annealing at 58°C for 30 sec and extention at 72°C for 1 min 30 sec. Final extension at 72°C was carried out for 10 min. The PCR product was run on a 2% agarose gel (section 2.4.1), visualised, and recorded as described in section 2.4.1. Primer pairs designed to amplify TGFβ1 receptor type 1 and GAPDH genes are given in Table 3.10

Amplicon	Accession Number	Forward Primer	Reverse Primer	Annealing Temp °C	Product size (bp)
TGFβ1 R1	NM_004612	ACTATCGT AAAGTCAT CACCTGGC	TCCTCTTC ATTTGGCA CTCGA	59	150
GAPDH	NM_002046	CTGCACCA CCAACTGC TTAGC	ATGCCAGT GAGCTTCC GTTC	58	205

Table 3.10: Primer sequences and product sizes

3.2.5 Q-RT-PCR

The relative levels of expression of SMAα, IGFBP-1, PR and DKK in ESC and PR in T47D cells, both control and treated cells (Tables 3.3 – 3.9), were determined using quantitative RT-PCR (TaqMan) using random primed cDNA as detailed in section 2.4.2.

Results were analysed as in section 2.4.2. Primer pairs and probes specific to each gene are given in Table 3.11.

Amplicon	Accession Number	Forward Primer	Reverse Primer	Probe
IGFBP-1	M59316	CACAGGAG ACATCAGG AGAAGAAA	ACACTGTCT GCTGTGAA AAATCCAT	TTCCAAATT TTACCTGCC AAACTGCAA CAA
Progesterone Receptor (nuclear)	NM_000926	CAGTGGGC GTTCCAAA TGA	GGTGGAAT CAACTGTA TGTCTTGA	AGCCAAGCC CTAAGCCAG AGATTCACT TT
DKK	AB020315	GGAATAAG TACCAGAC CATTGACA AC	GGGACTAG CGCAGTAC TCATCA	ACCAGCCGT ACCCGTGCG CA
SMAα	NM_001100	GTGTTGCC CCTGAAGA GCAT	ATTTTCTCC CGGTTGGC CTT	ACCCTGCTC ACGGAGGC ACCCCT

Table 3.11: Primer and probe sequences

3.2.6 Transient transfection

Transient transfections were performed using a reporter construct containing a PRE, ARE, GRE or ERE-linked to luciferase (section 2.5.1). A β-galactosidase control vector, (PCH110, Pharmacia), was co-transfected in all cases and used to measure β-galactosidase to control for transfection efficiency (section 2.5.4). Transient transfections were performed using the calcium chloride method as described in section 2.5.2. All transfections were performed in triplicate, using DCC media, a 1:1 mixture of DMEM and Ham's F-12 containing 5% FCS that had been depleted of steroids by treatment with dextran-coated charcoal, 100 U/ml penicillin, and 100

μg/ml streptomycin, and supplemented with 10^{-9} M 17ß-oestradiol and 1 μg/ml insulin (section 2.5.2).

Six hours after transfection the media was replaced with 2 % FCS DCC. The experiments were performed as detailed in Tables 3.12 – 3.15. The cells were harvested 48 h after treatment for analysis with a luciferase assay (section 2.5.3). Tranfection efficiency was analysed with a β-galactosidase assay (section 2.5.4). The samples were "normalised" by dividing each sample's luciferase reading with the corresponding β-galactosidase reading. This numerical reading was used as a comparison against other samples.

Experiment 3.8: Does TGFβ1 interfere with the transactivation potential of PR?		
Treatment	Concentration	Incubation period
Control	N/A	48 h
MPA	1μM	48 h
TGFβ1	10 ng/ml	48 h
MPA +TGFβ1	1 μM +10 ng/ml	48 h

Table 3.12: Experimental regime for experiment 3.8

Experiment 3.9: Does TGFβ1 interfere with the transactivation potential of AR?		
Treatment	Concentration	Incubation period
Control	N/A	48 h
DHT	0.1 μM	48 h
TGFβ1	10 ng/ml	48 h
DHT +TGFβ1	0.1μM +10ng/ml	48 h

Table 3.13: Experimental regime for experiment 3.9

Experiment 3.10: Does TGFβ1 interfere with the transactivation potential of GR?		
Treatment	Concentration	Incubation period
Control	N/A	48 h
DEX	0.25 μM	48 h
TGFβ1	10 ng/ml	48 h
DEX +TGFβ1	0.25 μM +10 ng/ml	48 h

Table 3.14: Experimental regime for experiment 3.10

Experiment 3.11: Does TGFβ1 interfere with the transactivation potential of ER?		
Treatment	Concentration	Incubation period
Control	N/A	48 h
Oestradiol	0.1 μM	48 h
TGFβ1	10 ng/ml	48 h
Oestradiol +TGFβ1	0.1 μM +10 ng/ml	48 h

Table 3.15: Experimental regime for experiment 3.11

3.2.7 siRNA for SMAD 4

Non-decidualised or decidualised ESCs were transiently transfected using the calcium phosphate precipitation method, as previously described in section 2.5.2, with 50 nM of the siRNA reagents (Dharmacon) detailed in Table 3.16. The RNA and protein content was extracted 72 h later as described in sections 2.3.1 and 2.6.1 respectively.

Experiment 3.13: Does interfering with the SMAD signalling pathway inhibit TGFβ1-induced responses?		
Treatment	Concentration	Incubation period
Mock transfection	N/A	72 h
Control siRNA (SiGENOME SMARTpool) MPA +8-Br-cAMP	1 μM + 250 μM	72 h
SMAD 4 siRNA (SiGENOME SMARTpool) MPA +8-Br-cAMP	1 μM + 250 μM	72 h
Control siRNA (SiGENOME SMARTpool) MPA +8-Br-cAMP + TGFβ1	1 μM + 250 μM 10 ng/ml	72 h
SMAD 4 siRNA (SiGENOME SMARTpool) MPA+8-Br-cAMP +TGFβ1	1 μM + 250 μM 10 ng/ml	72 h

Table 3.16: Experimental regime for experiment 3.13

3.2.8 Western Blotting analysis

Total cellular protein was extracted from the experiments detailed in Tables 3.6, 3.7 and 3.16 using the method detailed in section 2.6.1 and quantified using the method of Bradford (section 2.6.2). The protein samples were separated on a gel as described

in section 2.6.3 and subsequently electrotransferred onto a PVDF membrane (section 2.6.4). After blocking with 15 ml of 4 % BSA + 4 % NaN_3, the membrane was incubated at 4°C overnight with an anti-PR mouse monoclonal antibody (directed against both isoforms, PR A and PR B) (Novocastra), diluted 1:200 in the blocking solution or anti-SMAD 4 mouse monoclonal antibody (Abcam), diluted 1.500 in the blocking solution. After 3 washes with TNS Tween, the membrane was incubated for 1 h at room temperature with the secondary antibody; a rabbit anti-mouse IgG (Sigma) diluted 1:5000 in TNS Tween. The membrane was subsequently washed 3 times in TNS Tween before developing with ECL plus detection kit (Amersham Life Sciences) for 20 min, as described in section 2.6.5.

3.2.9 IGFBP-1 ELISA

Media collected from experiment 3.1 was assayed for IGFBP-1 using a two-site sandwich ELISA as described in section 2.6.7.1. Plates were coated with the IGFBP-1 capture antibody (R & D Systems) (2 μg/ml, 100 μl per well) and incubated overnight at 4°C. The plates were washed prior to use. A standard curve, consisting of eight standards, was added in duplicate to each plate. The standards were prepared as follows: a stock solution of 8 ng/ml was used to produce a dilution series to ultimately obtain a bottom standard of 0.06 ng/ml. Each standard was thoroughly mixed before the next transfer. The undiluted standard served as the high standard (8 ng/ml) ELISA Buffer plus Tween (B+T) was used to dilute standards and antibodies to be used later in the ELISA. ELISA buffer plus tween served as the NSB. A quality control was made up at 700 pg/ml from the IGFBP-1 standard and added in duplicate to each plate. The NSB, standards, and media samples to be assayed were added in duplicate, 100 μl per well. Plates were sealed and incubated for 3 h on a plate shaker at room temperature. The plates were washed four times each and residual wash buffer removed by aspiration. The IGFBP-1 detection antibody (R & D Systems) (100 μl per well) was added at a concentration of 0.1 μg/ml and incubated at room temperature for 90 min on a plate shaker. Plates were washed and residual wash buffer removed as before. Streptavidin peroxidase conjugate (Roche) (0.125 U/ml, 100 μl per well) was added and the plates incubated for 20 min on a plate shaker at

room temperature. The plates were then washed as before and 200 µl of peroxidase substrate added per well. Plates were developed for 10 min before stopping the reaction with 50 µl of 2 N sulphuric acid per well. All plates were read on a plate reader at 450 nM. The computer program, Assay Zap™, was used to analyse the results and determine the concentration of IGFBP-1 in each sample by interpolation from the standard curve.

3.2.10 DKK ELISA

Media collected from experiment 3.6 was assayed for DKK using a two-site sandwich ELISA as described in section 2.6.7.1. Plates were coated with the DKK capture antibody (R & D Systems) (2 µg/ml, 100 µl per well) and incubated overnight at 4°C. The plates were washed prior to use. A standard curve, consisting of eight standards, was added in duplicate to each plate. The standards were prepared as described in section 3.2.9 for the IGFBP-1 ELISA. The NSB, standards, and media samples to be assayed were added in duplicate, 100 µl per well. The remainder of the assay was conducted as detailed in section 3.2.9.

3.2.11 Immunocytochemistry

Immunocytochemistry was performed on ESC using the protocol described in section 2.6.9.2. Immunocytofluorescence was also performed using the protocol described in section 2.6.10. The ESCs were cultured on 2-well chamber-well slides (Nalgene, Nunc) and treated as detailed in Table 3.7 when 70–80 % confluence was attained. Immunohistochemistry used Tris-buffered saline (TBS) and immunohistofluorescence used Phosphate-buffered saline (PBS) in washes and dilutions.

Cultured cells were washed with cold TBS/PBS (Sigma) and then fixed in 4 % NBF for 2 h. Thereafter, cells were washed with TBS/PBS and then permeabilised by incubating the slides for 20 min at room temperature with 0.2 % NP40 (Sigma), 1 % Bovine Serum Albumin (BSA) (Sigma, Poole), and 10 % nonimmune Goat Serum

(NGS) (NP40/BSA/NGS). Non-specific binding sites of the primary antibody were blocked by incubating the slides in a 1:5 dilution of NGS in TBS/PBS containing 5 % BSA (NGS/TBS or PBS/BSA). Endogenous avidin/biotin was blocked using a commercially available avidin-biotin blocking kit (Vector Laboratories Ltd,) then washed twice with PBS. Slides were incubated at 4°C overnight in a 1:40 dilution of mouse monoclonal anti-human PR A+B (Novocastra Laboratories Ltd, Newcastle-Upon-Tyne, UK) or mouse monoclonal anti-human p-SMAD 2 (Abcam, Cambridge, UK) made up in NGS/TBS or PBS/BSA. After washing once with TBS/PBS Tween and once with TBS/PBS for 5 min each, PR antibody binding was detected by applying a 1:500 dilution of biotinylated goat antimouse antibody (Dako UK Ltd) in NGS/TBS/BSA, followed by an avidin/biotin horseradish peroxidase complex (Dako UK Ltd), for 60 min and 30 min respectively, at room temperature. Finally, antigenic sites were visualised by 3, 3-diaminobenzidine (Dako UK Ltd) before counterstaining in Harris's hematoxylin, dehydrating, and mounting with Pertex mountant. p-SMAD 2 binding was detected by applying a 1:500 dilution of biotinylated goat antimouse antibody (Dako UK Ltd) in PBS followed by streptavidin 546 (Molecular Probes) for 60 min each at room temperature. After washing the slides as described above, cells were counterstained with DAPI (1:200 in PBS) for 10 min at room temperature. The slides were mounted under a glass coverslip using Permafluor™ mounting medium.

3.2.12 Statistical analysis

Prior to statistical analysis, all data was tested for either Gaussian or non-parametric distribution. Where appropriate, values were presented as means ± S.E.M. Comparison of the different parameters for the various treatment groups was determined by repeated measures analysis of variance (ANOVA). Significant differences were assigned using Kruskal-Wallis post hoc test. The criterion for significance for all tests was set at $p < 0.05$. Specific software was used to assist in the data analysis (GraphPad Prism).

3.3 Results

3.3.1 Expression of TGFβ receptors in non-decidualised and decidualised stromal cells

Before embarking on a series of experiments to determine the impact of TGFβ1 on gene expression in non-decidualised and decidualised stromal cells, the presence of TGFβ receptors had to be ascertained. It was also necessary to ensure that the ESCs that had been decidualised *in vitro* were indeed expressing the classical decidualisation marker, IGFBP-1.

Successful *in vitro* decidualisation was assessed by measuring the IGFBP-1 mRNA expression and protein release in the decidualised ESCs compared with the non-decidualised ESCs. Non-decidualised cells are shown in panel A (Figure 3.1) and *in vitro* decidualised cells are shown in panel B (Figure 3.1). After decidualisation the cells become more rounded in appearance and appeared to have an accumulation of secretory products on the cell surface. Consistent with successful decidualisation, expression of IGFBP-1 mRNA was significantly upregulated in decidualised cells as compared with the non-decidualised samples ($p<0.05$, $n=4$) (Figure 3.2). This significant increase in IGFBP-1 mRNA was mirrored in the amount of IGFBP-1 protein release as detected with a two-site sandwich ELISA ($p<0.001$, $n=4$) (Figure 3.3).

Expression of TGFβ1 receptor type 1 (TGFβ1R1) mRNA was detected in both non-decidualised and decidualised ESCs (Figure 3.4, panel A). TGFβ1R1 mRNA expression appears to be more pronounced in decidualised ESCs (samples 1, 3, 5 and 7) as compared with non-decidualised ESCs (samples 2, 4, 6 and 8) in the semi-quantitative PCR. Panel B depicts the same samples probed for GAPDH, which was used as an internal control for integrity of the RNA samples) however this was not seen to be significant when quantitative RT-PCR (TaqMan) was conducted on the same samples (Figure 3.5).

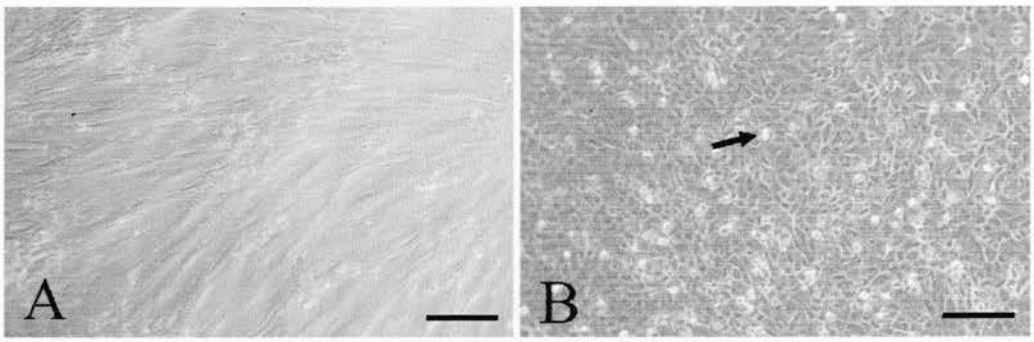


Figure 3.1: Photograph of non-decidualised (Panel A) and decidualised ESCs after 72 h treatment with 8-Br-cAMP and MPA (Panel B). The arrowhead highlights a cell with visible secretory products. Scale bars represent 100μm.

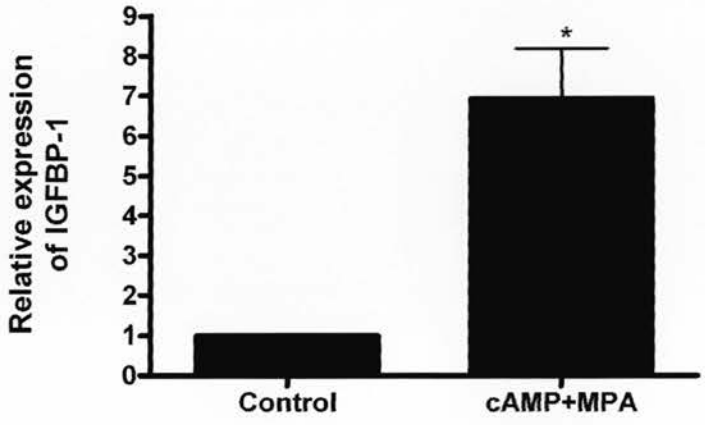


Figure 3.2: Relative expression of IGFBP-1 mRNA in non-decidualised and decidualised ESCs. Cells were either untreated or decidualised *in vitro* with 8-Br-cAMP and MPA for 72 h. IGFBP-1 mRNA was analysed by Q-RT-PCR. Results are ± SEM. ($p<0.05$) n= 4 endometrial samples.

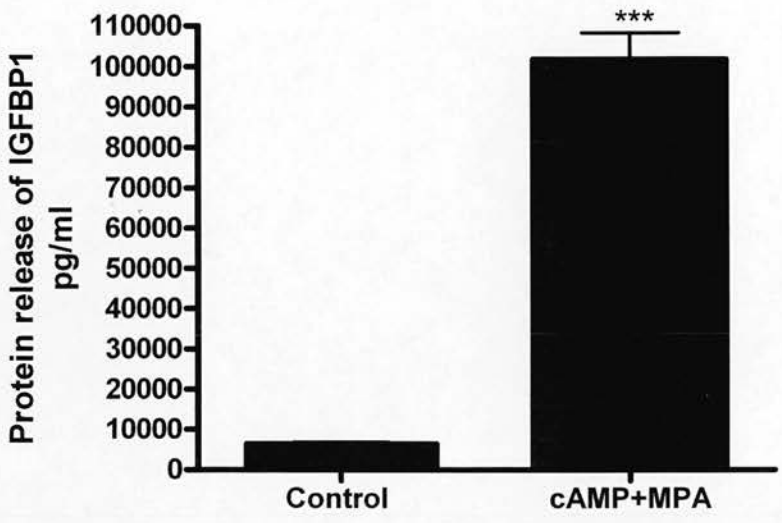


Figure 3.3: Quantitative analysis of IGFBP-1 protein release in non-decidualised and decidualised ESCs. Cells were either untreated or decidualised *in vitro* with 8-Br-cAMP and MPA for 72 h. IGFBP-1 protein release was analysed by ELISA. Results are ± SEM. ($p<0.001$) n= 4 endometrial samples.

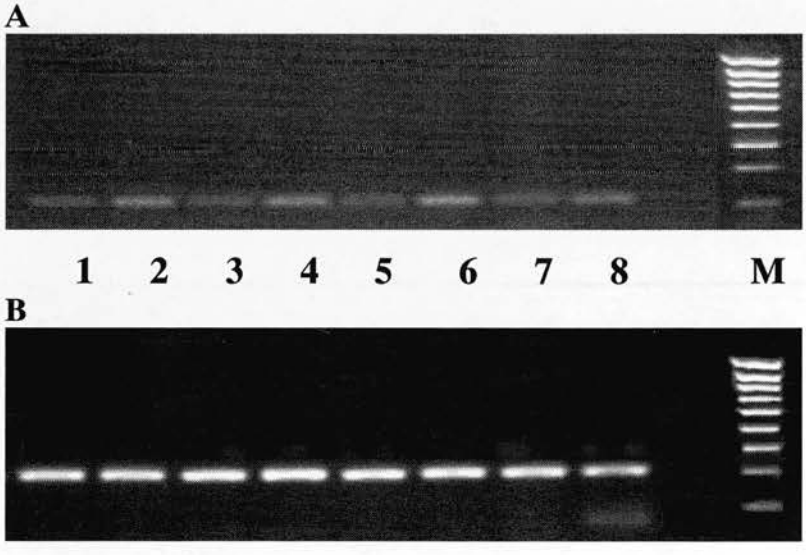


Figure 3.4: Expression of TGFβ1 R1 (150 bp) in non-decidualised and decidualised ESCs. Cells were either untreated or decidualised *in vitro* with 8-Br-cAMP and MPA for 72 h. The cultures were harvested and total cellular RNA probed for the expression of TGFβ1 R1 (Panel A) and GAPDH (205bp) (Panel B), the housekeeping gene. Sample M represents DNA hyperladder. Samples 1, 3, 5 and 7 depict non-decidualised ESCs. Samples 2, 4, 6 and 8 depict *in vitro* decidualised ESCs.

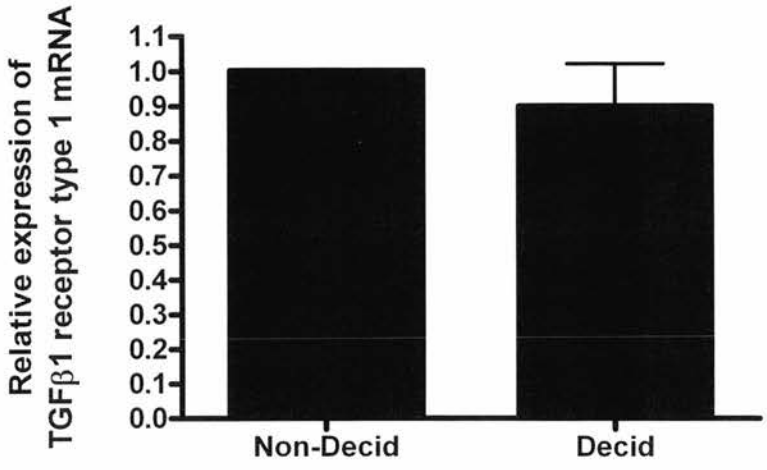


Figure 3.5: Relative expression of TGFβ1 R1 mRNA in non-decidualised and decidualised ESCs. Cells were either untreated or decidualised *in vitro* with 8-Br-cAMP and MPA for 72 h. TGFβ1R1 mRNA was analysed by Q-RT-PCR. Results are ± SEM n = 4 endometrial samples.

3.3.2 Identification of the most potent TGFβ isoform evoking an inbititory effect on PR mRNA

To address the question of the potency of TGFβ1 in comparison with the other TGFβ isoforms, which have previously been localised within the endometrium (Arici, MacDonald et al. 1996; Casslen, Sandberg et al. 1998), ESCs, decidualised *in vitro*, were treated for 72 h with TGFβ1, TGFβ2 or TGFβ3 (10 ng/ml). TGFβ1 alone evoked a significant decrease in PR, as compared with TGFβ2 and TGFβ3 ($p<0.001$, n = 6) (Figure 3.6). TGFβ2 and TGFβ3 were without significant effect, but displayed a mild trend towards reduction.

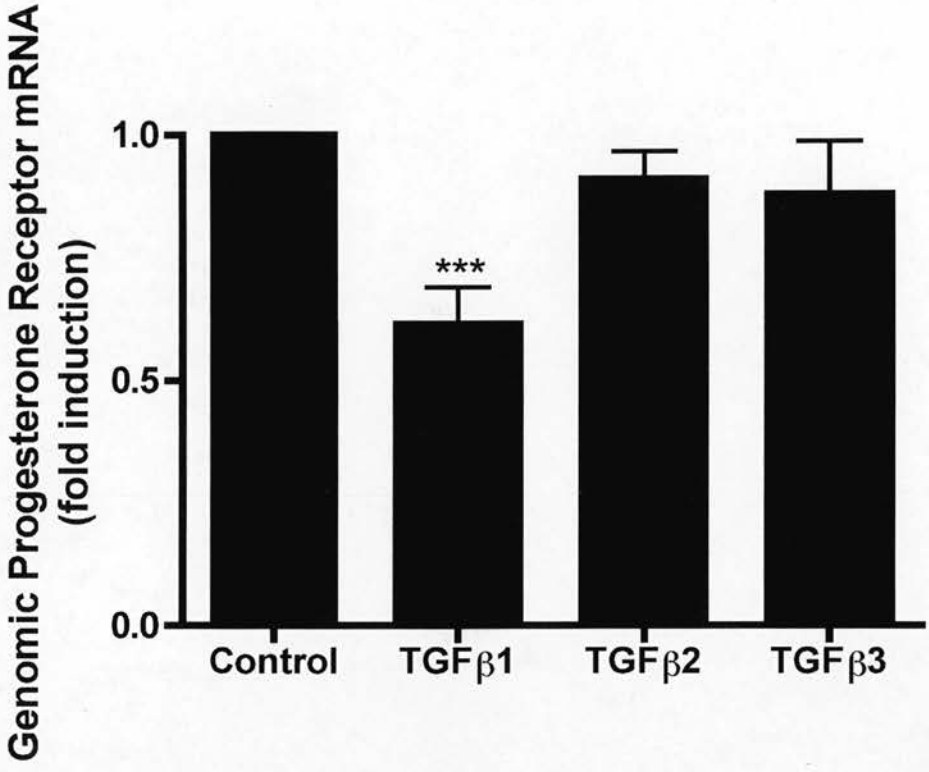


Figure 3.6: TGFβ1 is the most potent isoform localised within the endometrium. Cultured ESC, decidualised *in vitro* +/- either TGFβ1, TGFβ2, or TGFβ3 (all at 10 ng/ml), for 72 h. TGFβ1 reduces expression of mRNA nuclear PR in a significant manner ($p<0.001$). TGFβ2 and TGFβ3 show no significant effect. Results are ± SEM. n = 6 endometrial samples.

3.3.3 TGFβ1 upregulates SMA in both non-decidualised and decidualised ESCs

To address if TGFβ1 was acting as expected on ESC to induce a myofibroblast phenotype, ESC were cultured in the presence or absence of TGFβ1 (10 ng/ml) (Figure 3.7) or were cultured, decidualised *in-vitro* with 8-Br-cAMP 0.5 mM and MPA 1 μM (decidualising medium) for 6 d and then further cultured with decidualising medium to maintain the decidualised phenotype with or without the addition of TGFβ1 for up to 72 h (Figure 3.8). 10 ng/ml TGFβ1 up-regulated expression of SMAα mRNA in both non-decidualised ESC (Figure 3.7) ($p<0.01$, n = 9) and decidualised ESC (Figure 3.8) ($p<0.001$, n = 9) after 36 h of exposure. This

upregulation of SMAα continued in a time-dependent manner with a significant increase in SMAα observed at 48 h ($p<0.001$, n = 9) and 72 h ($p<0.001$, n = 9) in both non-decidualised (Figure 3.7) and decidualised cells (Figure 3.8) as compared to unstimulated, time-matched controls.

To elucidate a possible correlation between SMAα and TGFβ1, expression of mRNAs in control ESCs was measured. Cells were cultured for 72 h in serum-free medium without treatment. A significant positive correlation between endogenous SMAα and endogenous TGFβ1 mRNA expression ($p<0.05$, n = 9) was detected (Figure 3.9).

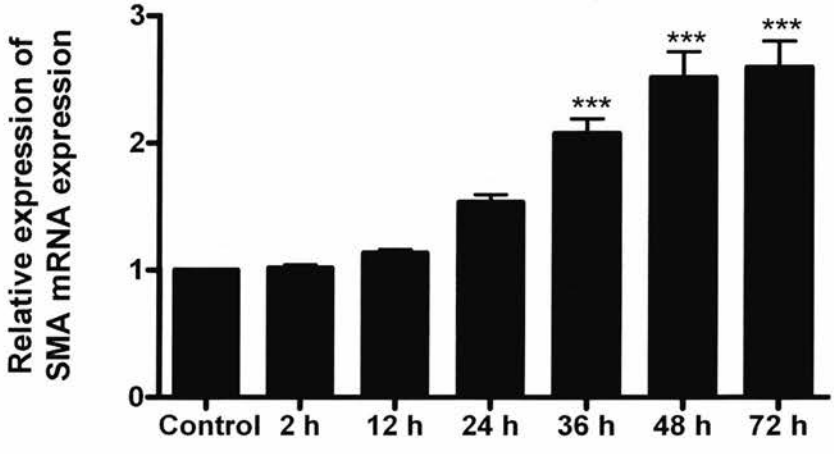


Figure 3.7: Time course of SMA mRNA expression in cultured ESC ± TGFβ1 measured by Q-RT-PCR. TGFß1 upregulates SMA mRNA in a time-dependent manner in primary ESC. Results are ± SEM. ($p<0.001$) n = 9 endometrial samples.

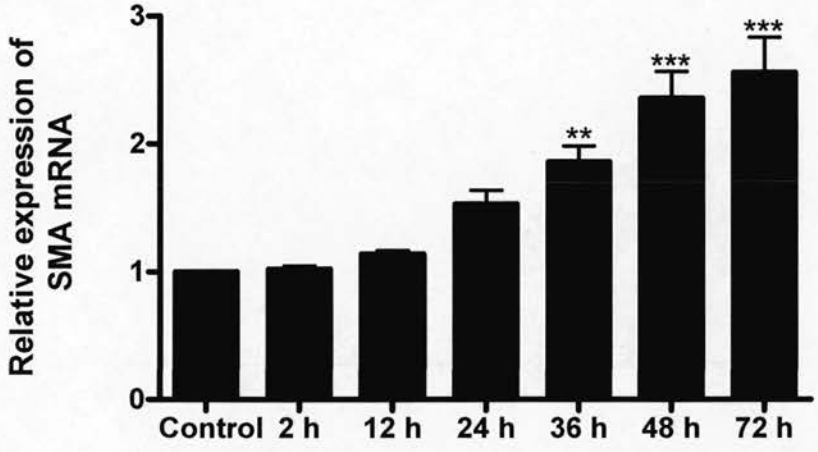


Figure 3.8: Time course of SMA mRNA expression in decidualised ESC +/-TGFβ1 measured by Q-RT-PCR. TGFβ1 increases expression of SMA mRNA in a time dependent manner. Results are ± SEM. ($p<0.01$, $p<0.001$) n = 9 endometrial samples.

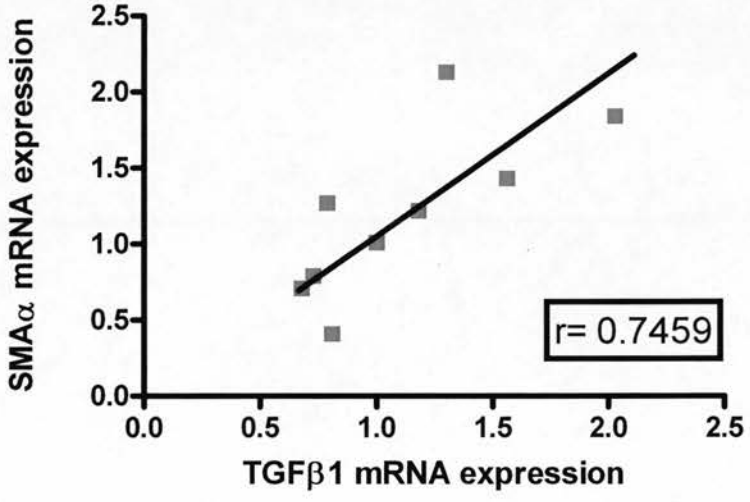


Figure 3.9: Correlation of SMAα mRNA expression with TGFβ1 mRNA expression in control ESCs. Cells were cultured for 72 h in serum free media. Correlation coefficient r = 0.7459. Significant correlation ($p<0.05$). n = 9 endometrial samples.

3.3.4 TGFβ1 downregulates PR in both non-decidualised and decidualised ESC in a time dependent manner

3.3.4.1 Non-decidualised ESC

To address the role of TGFβ1 regulation of ESC PR expression, ESC were cultured in the presence or absence of oestradiol (0.1 μM) and/or TGFβ1 (10 ng/ml) for up to 72 h. Results are presented in Figure 3.10 ; 10 ng/ml TGFβ1 up-regulated expression of PR mRNA in non-decidualised ESCs 4-fold ($p<0.001$, $n = 6$) after only 2 h of exposure, however, this increase was not maintained and PR mRNA was comparable to control cells. Incubation with TGFβ1 for 48 h and 72 h resulted in a significant downregulation in the level of PR mRNA expression as compared to unstimulated, time-matched controls ($p<0.05$, $n = 6$) (Figure 3.10). Treatment with oestradiol significantly up-regulated PR mRNA expression, in a time-dependent manner as compared to unstimulated, time-matched controls ($p<0.001$, $n = 6$) (Figure 3.10). Inclusion of TGFβ1 negated the oestradiol-induced response (Figure 3.10). The changes in mRNA expression were mirrored in PR protein expression, analysed by Western blotting (gel not shown). Densometric analysis of PR compared with β-actin (72 h treatment, Figure 3.11) revealed that TGFβ1 significantly downregulates ($p<0.001$, $n = 3$) and E_2 upregulates ($p<0.001$, $n = 3$) PR expression compared to control (Figure 3.11). Cells treated with E_2 plus TGFβ1 contained concentrations of PR comparable to those of untreated controls (Figure 3.11).

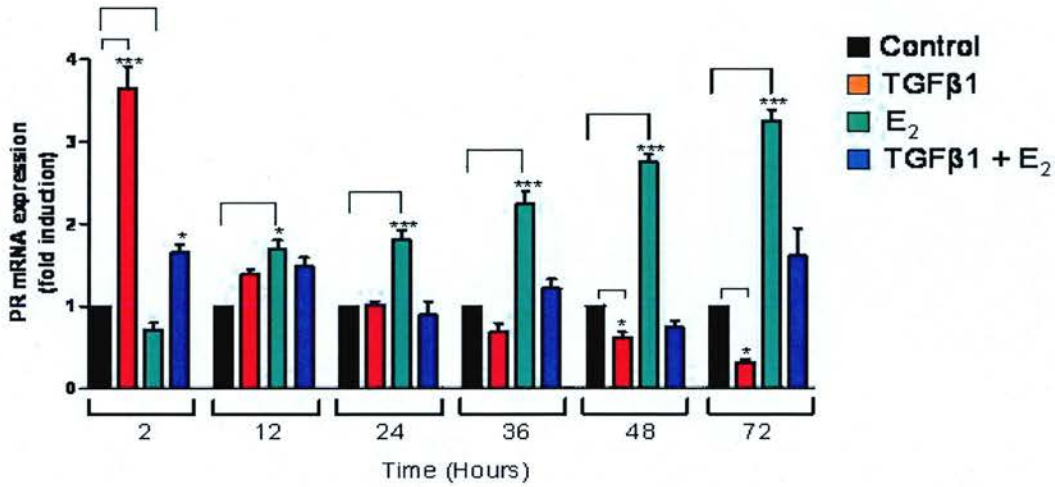


Figure 3.10: Time course of PR mRNA expression in cultured ESC ± TGFβ1 ± oestradiol measured by Q-RT-PCR. Oestradiol increases whilst TGFβ1 reduces expression of PR mRNA in a time dependent manner in primary ESC. Results are ± SEM. ($p<0.05$). n = 6 endometrial samples.

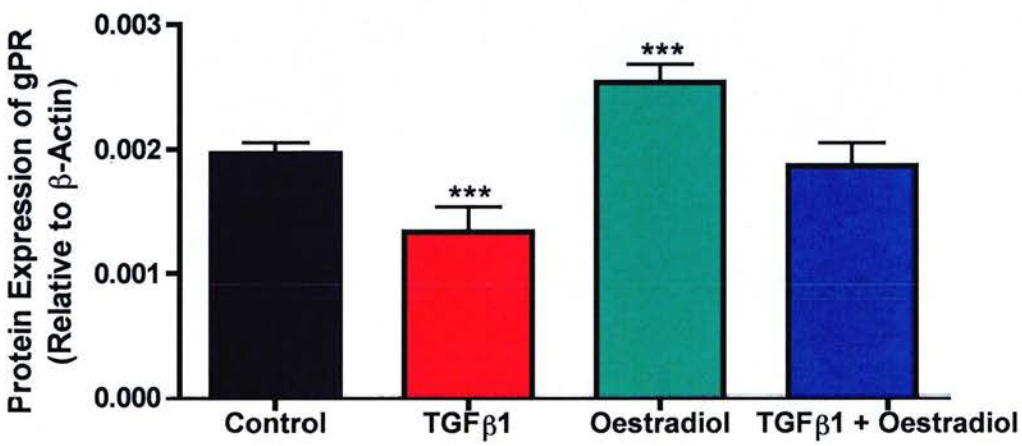


Figure 3.11: Densometric analysis of PR protein expression in whole cell lysates from cultured primary ESC ± TGFβ1 (10 ng/ml) ± oestradiol (0.1 μM), subjected to Western blotting analysis. The cultures were harvested and total cellular protein probed for the expression of PR A + B (120 kDa and 90 kDa respectively) and β Actin (48 kDa) the housekeeping gene. Results are ± SEM. ($p<0.001$) n = 3 endometrial samples.

3.3.4.2 Decidualised ESC and T47D cells

To further address the role of TGFβ1 in regulation of ESC PR expression, ESC were cultured, decidualised *in vitro* with 8-Br-cAMP (0.5 mM) and MPA (1 μM) (decidualising medium) for 6 d and then further incubated with decidualising medium with or without the addition of TGFβ1 for a maximum of 72 h. Results are presented in Figure 3.12, Graph A. TGFβ1 upregulated expression of PR mRNA in decidualised ESCs 2-fold ($p<0.001$, $n = 6$) after only 2 h of exposure, however, at 24 h and 36 h this up-regulation was not observed (Figure 3.12, Graph A). At 72 h PR mRNA expression levels were significantly downregulated 2-fold as compared to unstimulated controls ($p<0.05$, $n = 6$) (Figure 3.12, Graph A). Similar results were observed in T47D cells (Figure 3.12, Graph B). Immunoexpression of PR was localised to nuclei staining for in primary ESC (Figure 3.13), Panels I and II depict ICC on both control and TGFβ1–treated cells. Panel III and IV depict ICC on T47D cells, again on control and TGFβ1-treated cells. Comparison of staining intensities indicates a decrease of immunoreactive PR in the nuclei of both cell types. Representative examples are shown (Figure 3.13).

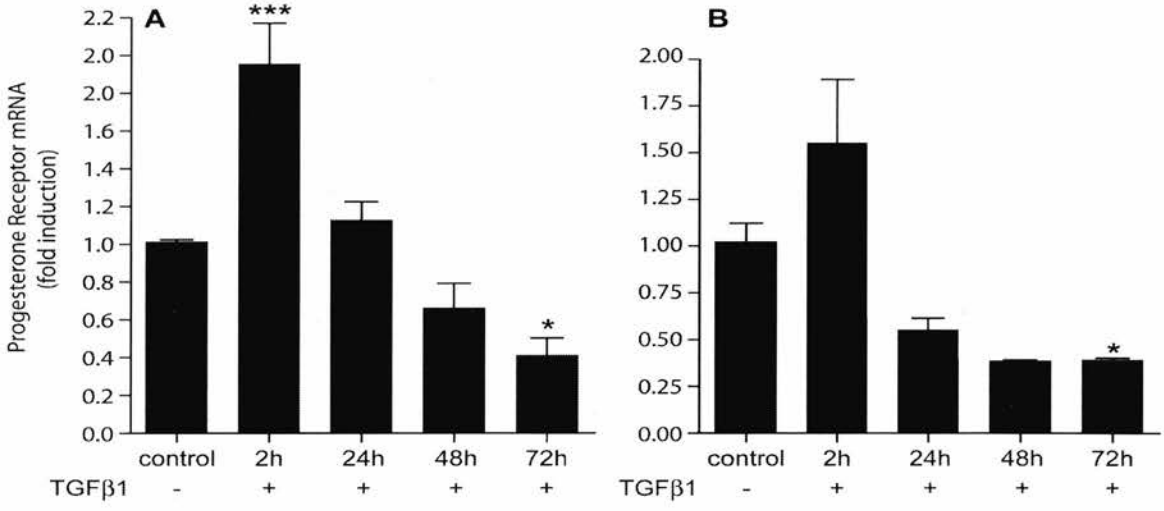


Figure 3.12: Time course of PR mRNA expression in A: Cultured ESC, decidualised *in-vitro* +/-TGFβ1 and B: T47D cells +/- TGFβ1 measured by Q-RT-PCR. TGFβ1 reduces expression of PR mRNA in a time dependent manner in both primary ESC and T47D cells. Results are ± SEM. ($p<0.05$) $n = 6$ endometrial samples.

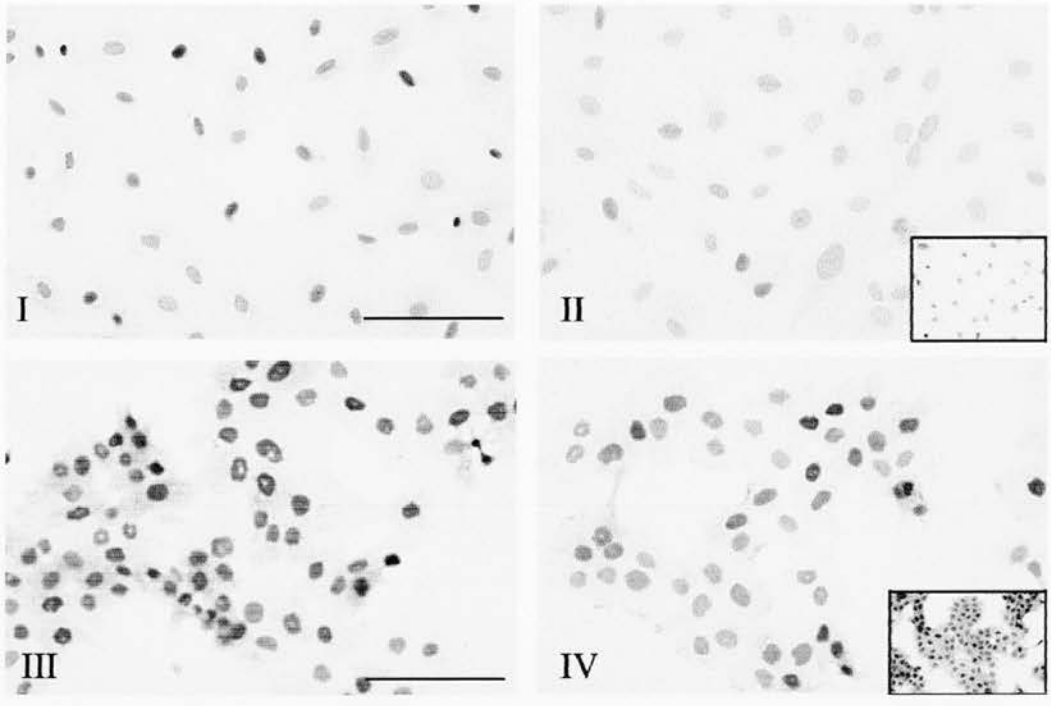


Figure 3.13: Immunohistochemical analysis of PR expression in control and TGFβ1-treated ESC and T47D cells. PR immunostaining in I; ESC decidualised *in vitro*, II; ESC decidualised *in vitro* + TGFβ1 (10 ng/ml), III; T47D cells, IV; T47D cells + TGFβ1 (10 ng/ml). Inserts show negative controls (no primary antibody). Scale bars represent 100 μm.

3.3.4.3 Comparison of non-decidualised and decidualised ESCs

Protein expression of PR and PIAS γ in non decidualised and decidualised ESCs was analysed by Western blotting (Figure 3.14). On Western blots we observed reduced expression of PR A and PR B following treatment of decidualised ESCs with TGFβ1 (Figure 3.14). This response appears more marked in cells exposed to decidualising agents for 72h (Figure 3.14). No response was observed in non-decidualised ESCs. This data is unconvincing and may be an abstract result; therefore, further Western blots should be performed on additional samples with densometric analysis conducted to quantify any change in protein expression between treatment groups. It would appear that TGFβ1 treatment potentiates protein expression of PIASγ in a time dependent manner in both non-decidualised and decidualised cells (Figure 3.14).

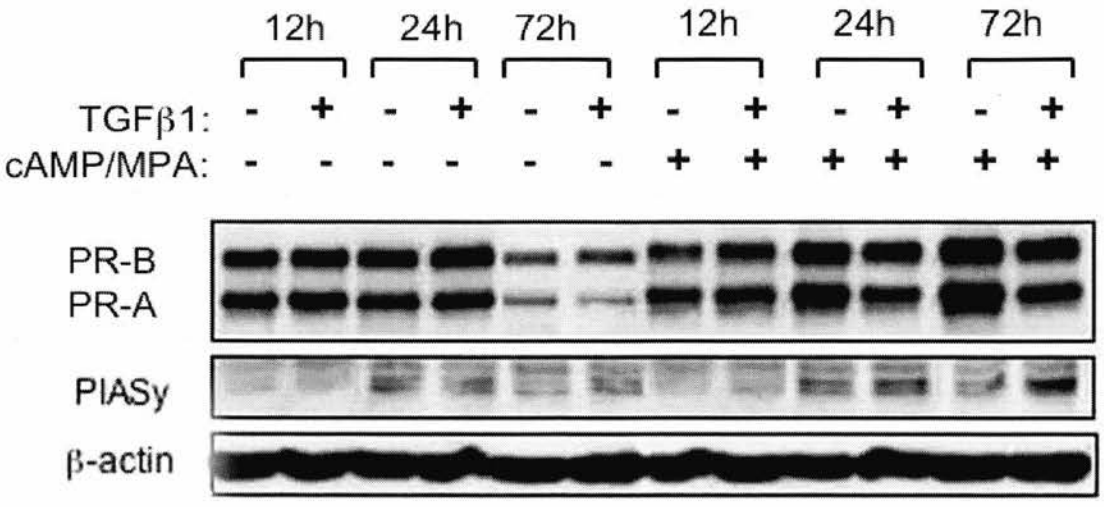


Figure 3.14: Whole cell lysates from untreated primary ESC or cultures decidualised *in vitro* for 12, 24 or 72 h, +/- TGFβ1 (10 ng/ml), were subjected to Western blotting analysis. TGFβ1 treatment reduced protein expression of both PR A and PR B in decidualised ESC and increased protein expression of PIASγ in both non-decidualised and decidualised ESC. Marius Jones performed this nuclear protein extraction and Western Blot in Jan Brosens lab group.

3.3.5 TGFβ1 does not affect the transactivation potential of steroid receptors

In order to elucidate if the TGFβ1 mediated-signalling can have a direct effect on the progesterone response element (PRE) or response elements for other steroid receptors, (AR, GR and ER) a luciferase promoter/reporter assay was conducted. The presence of an appropriate steroid receptor-agonist, but not addition of TGFβ1, induced reporter gene expression for all steroid response elements (Figure 3.15). Co incubation with steroid agonist and TGFβ1 did not have a significant impact on reporter gene activation (Figure 3.15).

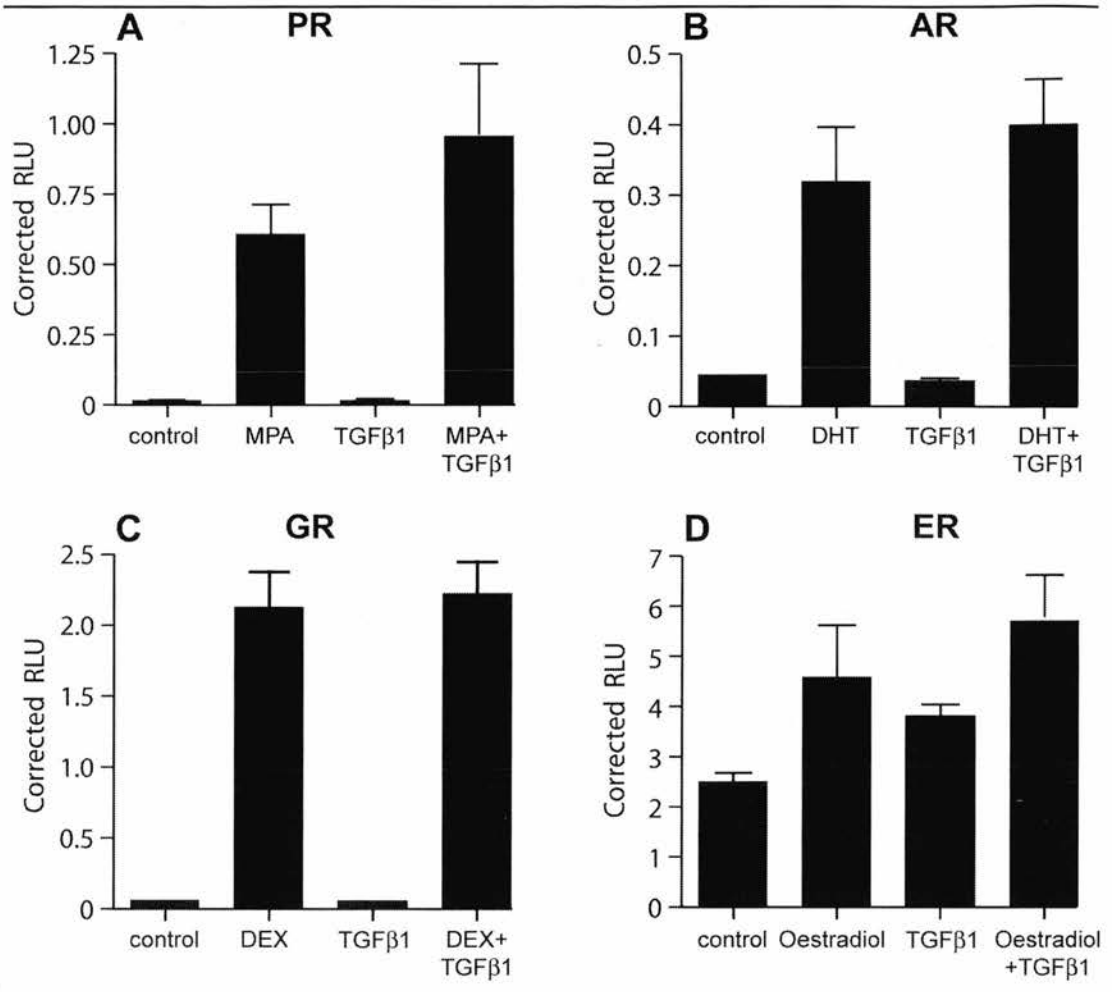


Figure 3.15: ESCs were transiently transfected with either, A: PRE-luc, B: ARE-luc, C: GRE-luc or D: ERE-luc expression vector and cells were treated with +/- MPA (PRE), DHT (ARE), DEX (GRE), E (ERE) or +/- TGFβ1. Results are ± SEM n = 3 endometrial samples. TGFβ1 was without effect, either alone or in combination with the steroid-specific agonist, on induction of reporter gene activation for all response elements.

3.3.6 TGFβ1 downregulates DKK in non decidualised and decidualised stromal cells

3.3.6.1 Non-decidualised ESCs

To address the question as to whether TGFβ1 interacts with the wnt-signalling antagonist DKK in non decidualised stromal cells, ESCs were cultured in the presence or absence of TGFβ1 (10 ng/ml) for up to 72 h. Results are presented in Figure 3.16; 10 ng/ml TGFβ1 significantly downregulated expression of DKK mRNA after only 2 h of exposure ($p<0.001$, $n = 5$), however, at 12 h and 24 h this downregulation was not observed (Figure 3.16). At 36 h and 48 h after TGFβ1 exposure DKK mRNA expression levels were significantly downregulated as compared to unstimulated, time-matched controls ($p<0.05$ and $p<0.001$ respectively, $n = 5$) (Figure 3.16). At 72 h after treatment DKK mRNA levels were comparable with the unstimulated, time-matched controls (Figure 3.16). DKK protein release was also measured. Results are presented in Figure 3.17: 10 ng/ml TGFβ1 does not significantly inhibit DKK protein release until 24 h after treatment ($p<0.001$, $n = 5$) (Figure 3.17). This shows some discrepancy from mRNA data in Figure 3.16, although it can be explained by the normal mRNA and protein kinetics, in that any changes in mRNA levels would be detected prior to protein changes.

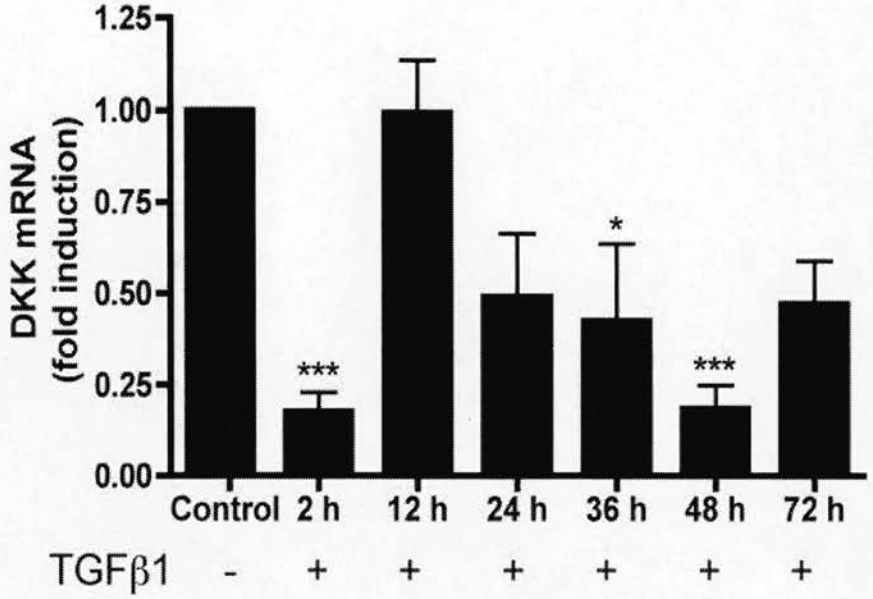


Figure 3.16: Time course of DKK mRNA expression in cultured ESC ± TGFβ1 measured by Q-RT-PCR. TGFβ1 downregulates DKK mRNA in a biphasic manner in primary ESC. Results are ± SEM. * = $p<0.05$, *** = $p<0.001$, n = 5 endometrial samples.

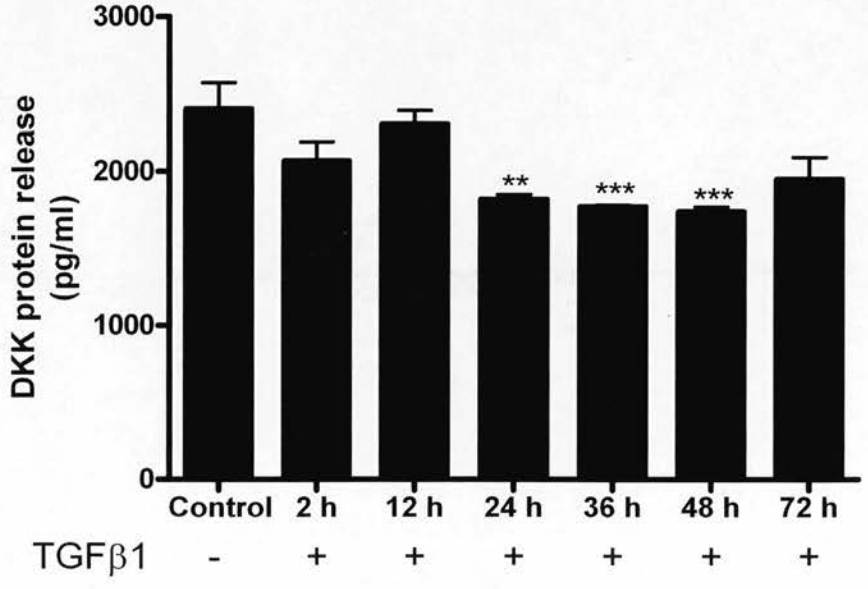


Figure 3.17: Time course of DKK protein release in cultured ESC +/- TGFβ1 measured by ELISA. TGFβ1 significantly reduces protein release of DKK over a period of 24 h, 36 h and 48 h (** = $p<0.01$, *** = $p<0.001$), but displays a return to control levels if cultured for 72 h or more. Results are ± SEM. n = 5 endometrial samples.

3.3.6.2 Decidualised ESC

To further address the role of TGFβ1 in regulation of DKK expression, ESC were cultured, decidualised *in vitro* with 8-Br-cAMP (0.5 mM) and MPA (1 μM) (decidualising medium) for 6 d and then further cultured with decidualising medium to maintain the decidualised phenotype with or without the addition of TGFβ1 for a maximum of 72 h. Results are presented in Figure 3.18. TGFβ1 significantly downregulates DKK mRNA expression over a period of 24 h ($p<0.05$, $n = 5$) (Figure 3.18) but displays a return to control levels if cultured for 48 h or more (Figure 3.18). Protein release was also measured in the same samples as shown in Figure 3.19: 10 ng/ml TGFβ1 significantly reduces protein release of DKK in a time-dependent manner over a period of 48 h ($p<0.001$, $n = 5$) (Figure 3.19). Downregulation of DKK is seen at 72 h also ($p<0.001$, $n=5$). It is also apparent from comparison of Figures 3.17 and 3.19 that DKK production is upregulated with decidualisation; non decidualised cells release approximately 2000 pg/ml DKK (Figure 3.17) whereas decidualised cells release approximately 8500 pg/ml DKK protein (Figure 3.19). This is further corroborated by results presented in Figure 3.21; treatment with decidualising medium significantly upregulated protein release of DKK after 72 h ($p<0.001$, $n = 5$) (Figure 3.21). No results are available for 36 h time point in decidualised cells as these cells became infected during the experiment. The remainder of cells were infection-free.

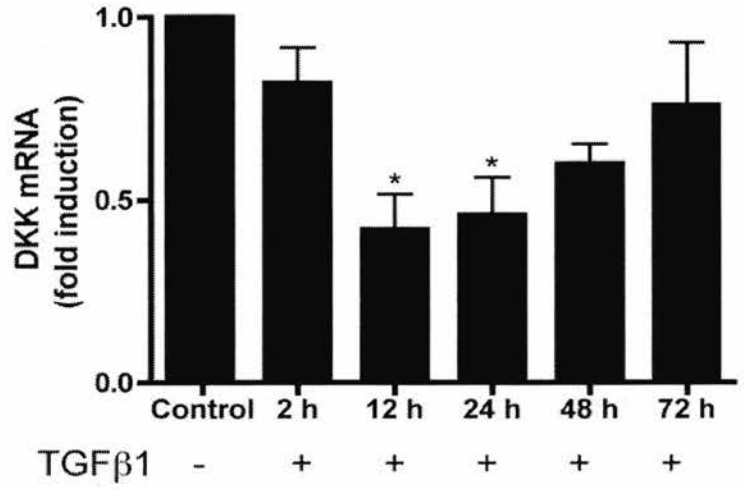


Figure 3.18: Time course of DKK mRNA expression in decidualised ESC +/-TGFβ1 measured by Q-RT-PCR. TGFβ1 reduces expression of DKK mRNA over a period of 24 h, but displays a return to control levels if cultured for 48 h or more. Results are ± SEM. ($p<0.05$) n = 5 endometrial samples.

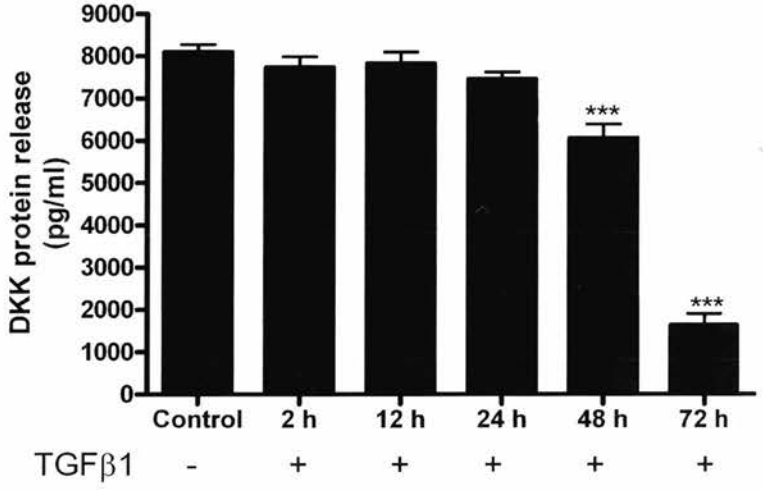


Figure 3.19: Time course of DKK protein release in cultured ESC, decidualised *in vitro* +/-TGFβ1 (10 ng/ml) quantified by ELISA. TGFβ1 significantly reduces protein release of DKK in a time-dependent manner over a period of 48 h ($p<0.001$). Downregulation of DKK is seen at 72 h also ($p<0.001$). Results are ± SEM. n = 5 endometrial samples.

3.3.7 Blocking endogenous TGFβ1 action

In an attempt to elucidate if blocking endogenous TGFβ1 production would negate the TGFβ1-induced responses, ESC were cultured in the presence and absence of decidualising medium for 6 d and then further cultured with decidualising medium to maintain the decidualised phenotype in the presence or absence of TGFβ1 (10 ng/ml) or anti-TGFβ1 antibody (1 μg/ml) for 72 h. As previously observed, TGFβ1 significantly downregulated expression of PR mRNA (Figure 3.20). In contrast, treatment with anti-TGFβ1 neutralises endogenous TGFβ1 and significantly increased expression of PR mRNA (Figure 3.20) ($p<0.001$, $n = 5$). Protein release of DKK was also measured; TGFβ1 significantly downregulated protein release of DKK (Figure 3.21). Treatment with anti-TGFβ1 neutralises endogenous TGFβ1 and significantly increased protein release of DKK (Figure 3.21) ($p<0.001$, $n = 5$). Treatment with the isotype control, Mouse IgG1, evoked no response in either PR mRNA expression or DKK protein release (Figures 3.20 and 3.21, respectively). TGFβ1 + anti-TGFβ1 was not included as a treatment as it would be extremely difficult to neutralise 10 ng/ml TGFβ1 and therefore any results obtained would be inconclusive.

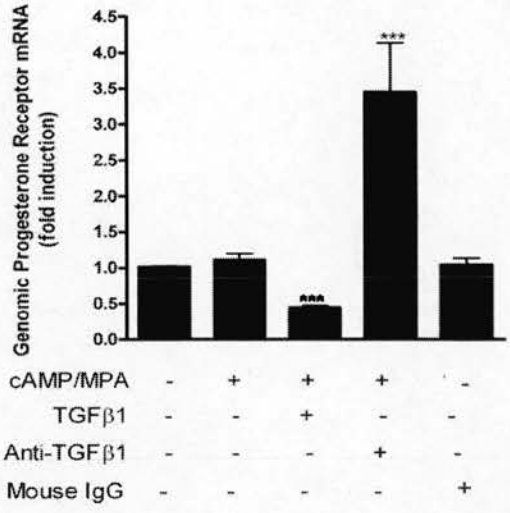


Figure 3.20: Cultured ESC were decidualised *in vitro* +/- TGFβ1-treatment. To confirm the specificity of the TGFβ1 response anti-TGFβ1 (1 μg/ml) or mouse IgG control was added, for 72 h. TGFβ1 reduces expression of mRNA nuclear PR, whilst anti-TGFβ1 antibody increases nuclear PR. Results are ± SEM. ($p<0.05$) n = 5 endometrial samples.

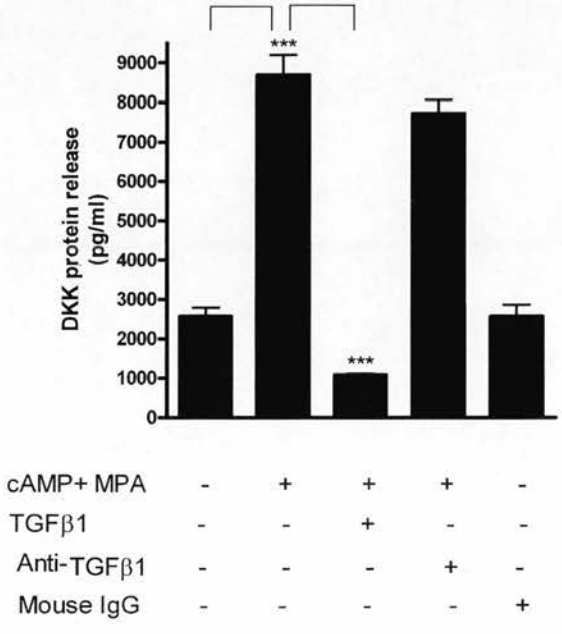


Figure 3.21: Cultured ESC were decidualised *in vitro* +/- TGFβ1-treatment. To confirm the specificity of the TGFβ1 response anti-TGFβ 1 (1 μg/ml) or mouse IgG control was added, for 72 h. TGFβ1 reduces protein expression of DKK, whilst anti-TGFβ 1 antibody increases DKK. Results are ± SEM ($p<0.05$). n = 5 endometrial samples.

3.3.8 TGFβ1 signals via the SMAD-signalling pathway in non-decidualised and decidualised ESC

To confirm that TGFβ1 was signalling via the SMAD-signalling pathway, primary ESC were cultured in the presence and absence of decidualising medium for 6 d and then further cultured with decidualising medium to maintain the decidualised phenotype in the presence or absence of TGFβ1 (10 ng/ml) or anti-TGFβ1 antibody (1 μg/ml) for 72 h, prior to measuring any changes in phosphorylated SMAD 2 (p-SMAD 2) protein expression. Changes in protein expression were detected by Western blotting and immunocytofluorescence (ICF) (Figures 3.22 and 3.23 respectively). Consistent with successful TGFβ–signalling mediated via the SMAD-signalling pathway, p-SMAD 2 protein expression is upregulated at every time point in both non-decidualised and decidualised ESC (Figure 3.22). This is in agreement with immunocytofluorescence staining (Figure 3.23). Panels I and III depict ICF on control cells, panel III is a larger viewfield of panel I. Panels II and IV depict ICF on TGFβ1-treated cells, panel IV is a larger view field of panel II. Deciphering staining intensities with the naked eye can be very subjective, for this reason the images were quantitatively analysed as described in section 2.6.10.11. Results are presented in Figure 3.24; 10 ng/ml TGFβ1 significantly upregulated p-SMAD 2 protein expression in primary ESC ($p<0.001$, $n = 3$).

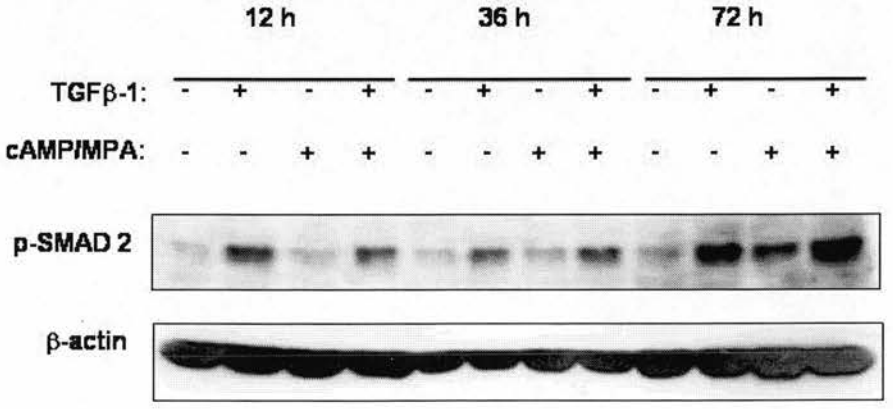


Figure 3.22: Nuclear lysates from untreated primary ESC or cultures decidualised *in vitro* for 12, 36 or 72 h, +/- TGFβ1 (10 ng/ml), were subjected to western blotting analysis. Marius Jones performed this nuclear protein extraction and Western Blot in Jan Brosens lab group. n = 5 endometrial samples.

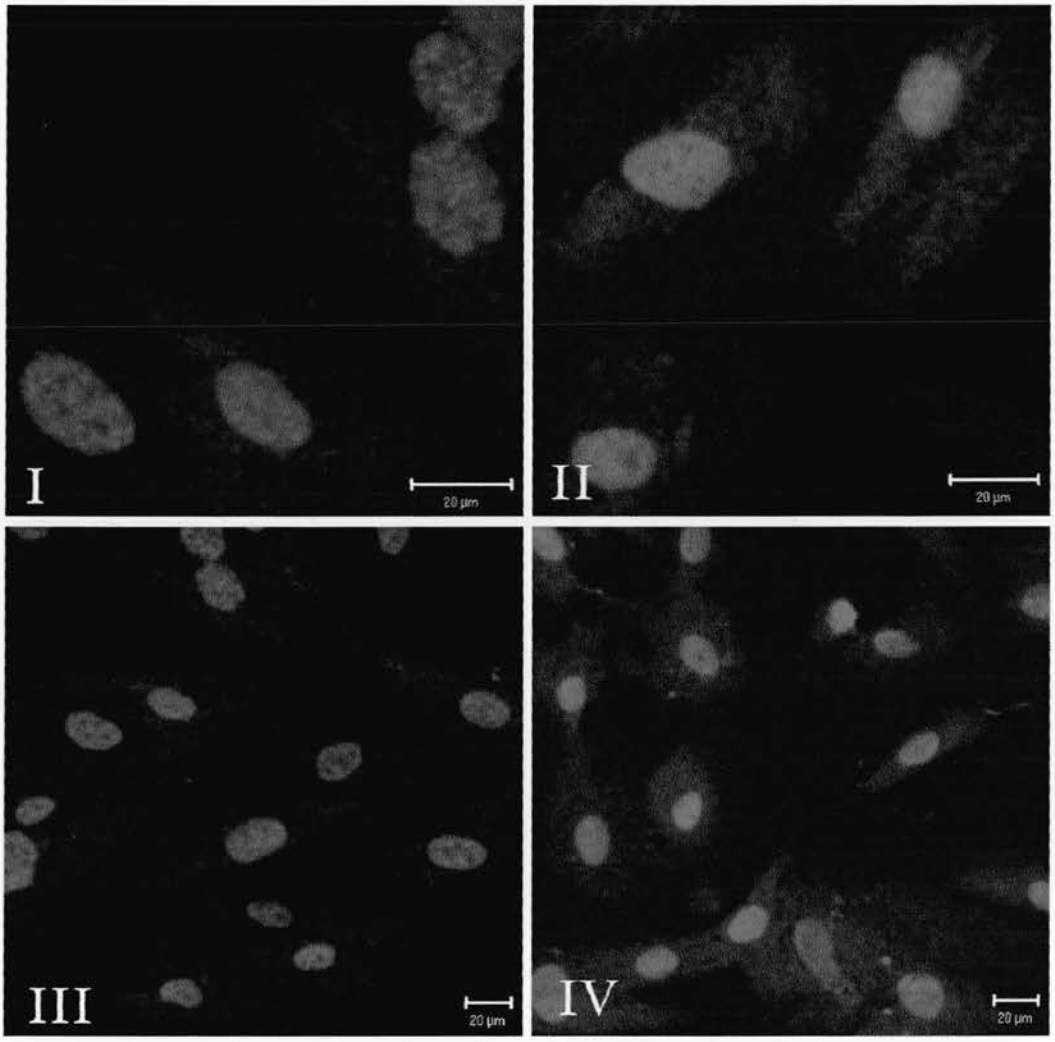


Figure 3.23: Immunocytofluorescence analysis of phosphorylated SMAD 2 (p-SMAD 2) expression in control and TGFβ1-treated ESC. p-SMAD 2 immunostaining in I; ESC without treatment, II; ESC + TGFβ1 (10 ng/ml), III; ESC without treatment, larger view field, IV; ESC + TGFβ1 (10 ng/ml), larger view field.

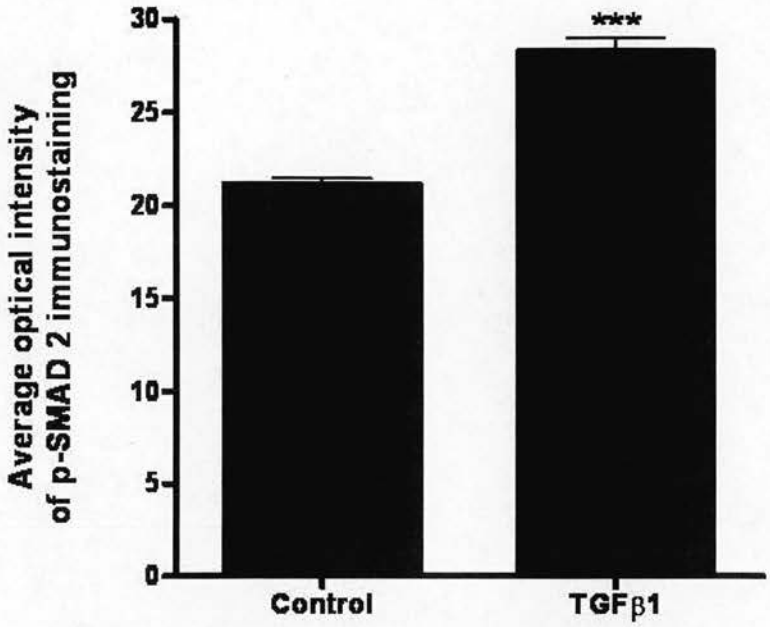


Figure 3.24: Quantitative analysis of average optical intensity of p-SMAD 2. 15 control and 15 TGFβ1- treated ESCs (10 ng/ml) were analysed from each endometrial biopsy. All settings were identical for control and treated cells. TGFβ1 significantly upregulated p-SMAD 2 protein expression ($p<0.001$). n = 5 endometrial biopsies.

3.3.9 Effect of abrogating the SMAD signalling pathway on TGFβ1- induced responses

SMAD 4 siRNA was used to investigate if selectively silencing the SMAD signalling pathway would abrogate the TGFβ1-induced responses. ESCs were transiently transfected as detailed in Table 3.16. Successful silencing was assessed by detecting the SMAD 4 mRNA and protein levels by non-quantitative PCR and Western blotting respectively. SMAD 4 mRNA was detected in all samples (Figure 3.25, Panel A). Sample 1 represents mock transfected cells. Sample 2 represents cells transfected with control siRNA (a non-targeting siRNA). Sample 3 represents cells transfected with SMAD 4 siRNA. Samples 2 and 3 were subsequently treated with decidualising stimuli, 8-Br-cAMP (0.5 mM) and MPA (1 μM). Sample 4 represents cells transfected with control siRNA, sample 5 represents cells transfected with SMAD 4 siRNA and subsequently treated with decidualising stimuli and TGFβ1 (10 ng/ml). SMAD 4 mRNA would appear to be more pronounced in response to TGFβ1

treatment, as seen in sample 4, than in any other sample (Figure 3.25). When SMAD 4 siRNA is introduced to the cell the TGFβ1-induced upregulation of SMAD 4 is not seen (sample 5), however, there is still evidence of mRNA present (Figure 3.25). Panel B shows the same samples analysed using primers directed against GAPDH, which was used as an internal control for integrity of the RNA samples. Protein analysis of the same treatments demonstrated effective silencing of SMAD 4 when cells were transfected with SMAD 4 siRNA (Figure 3.26). Treatment with TGFβ1 did not induce SMAD 4 protein expression in cells previously transfected with SMAD 4 siRNA (Figure 3.26).

To elucidate if silencing SMAD 4 would abrogate the TGFβ1-induced effect on PR mRNA levels were measured. Results are presented in Figure 3.27; interestingly treatment with cAMP and MPA evoked an increase in PR mRNA expression (Figure 3.27). This has not been observed previously. It is acknowledged that these data are derived from a single experiment and require further confirmation. A significant reduction in PR mRNA was observed in samples transfected with either the non-targeting siRNA or the SMAD 4-specific siRNA ($p<0.001$) (Figure 3.27), consequently, the treatments could not be analysed with regard to SMAD 4 dependent TGFβ-mediated PR downregulation. No significant reduction in PR mRNA was observed with samples transfected with SMAD 4 siRNA when compared to the non-targeting transfected samples (Figure 3.27). The transient transfection, treatment of the cells and Western blot was performed by Marius Jones in Jan Brosen's lab.

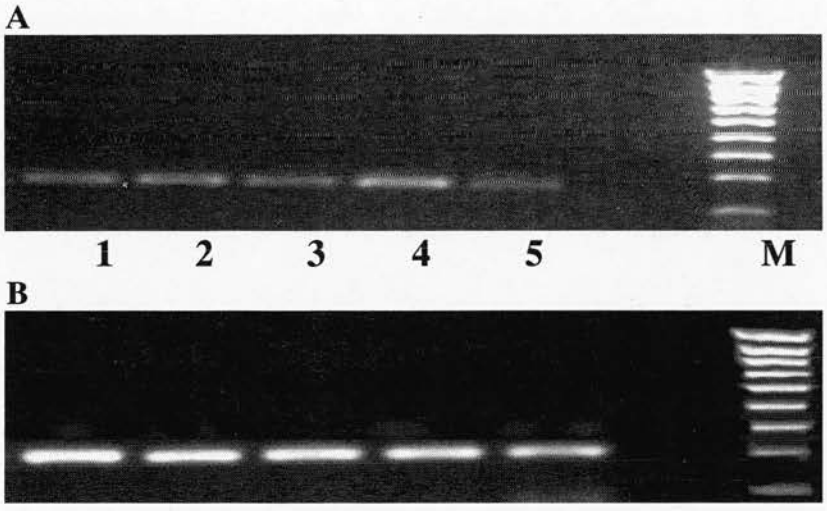


Figure 3.25: Semi-quantitative PCR of SMAD 4. Sample 1 represents mock transfected cells. Sample 2 represents cells transfected with control siRNA (a non-targeting siRNA). Sample 3 represents cells transfected with SMAD 4 siRNA. Samples 2 and 3 were subsequently treated with decidualising stimuli, 8-Br-cAMP (0.5 mM) and MPA (1 μM). Sample 4 represents cells transfected with control siRNA, sample 5 represents cells transfected with SMAD 4 siRNA and subsequently treated with decidualising stimuli and TGFβ1 (10 ng/ml). n = 1 endometrial sample.

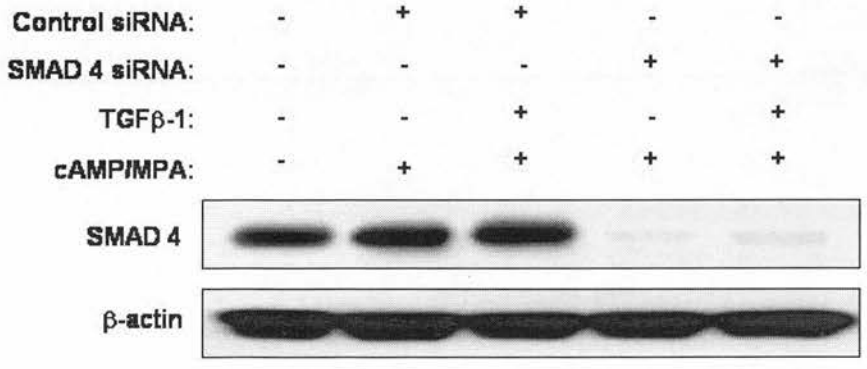


Figure 3.26: Whole cell lysates from primary ESC transiently transfected with either control siRNA or SMAD 4 siRNA subsequently cultured for 72 h, +/- 8-Br-cAMP (0.5 mM) and MPA (1 μM) +/- TGFβ1 (10 ng/ml), were subjected to Western blotting analysis. Marius Jones performed this protein extraction and Western Blot in Jan Brosens lab group.

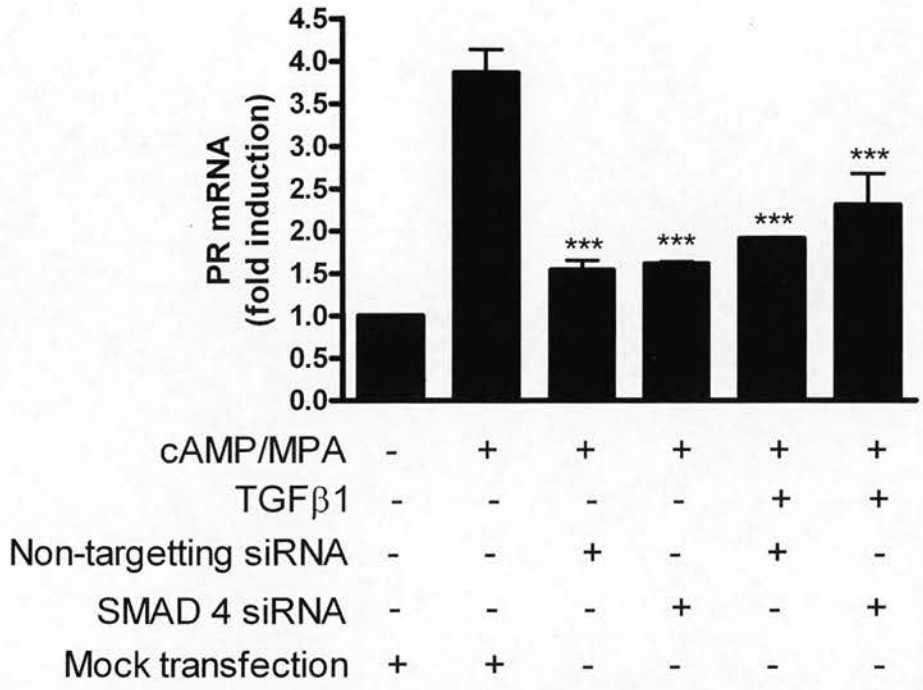


Figure 3.27: Cultured ESCs were transiently transfected with either control siRNA or SMAD 4 siRNA and subsequently cultured for 72 h, +/- 8-Br-cAMP (0.5 mM) and MPA (1 μM) +/- TGFβ1 (10 ng/ml). Treatment with decidualising stimuli evoked an increase in PR mRNA. A significant reduction was observed with samples transfected with the non-targeting siRNA and SMAD 4 siRNA, both treated with and without TGFβ1 ($p<0.001$). No significant difference was observed between samples transfected with the non-targeting siRNA and the SMAD 4 siRNA. n = 1 endometrial sample.

3.4 Discussion

The post-ovulatory rise in progesterone, acting via the nuclear progesterone receptor (PR) (Conneely, Lydon et al. 2000; Mulac-Jericevic, Mullinax et al. 2000), induces profound phenotypical and morphological remodelling of the E_2-primed endometrium resulting in decidualisation. Decidualisation is characterised by the transformation of elongated fibroblast-like phenotype of endometrial stromal cells (ESC) to a larger, spherical decidual cell coupled with the coordinate expression of decidualisation products such as IGFBP-1 (Bell, Jackson et al. 1991; Bryant-Greenwood, Rutanen et al. 1993). Several studies provide evidence that progesterone

interacts with other factors e.g. cAMP, to mediate its effect on differentiating endometrial stroma (Gellersen, Kempf et al. 1994; Brar, Frank et al. 1997; Brosens, Hayashi et al. 1999; Mote, Balleine et al. 2000; Mak, Brosens et al. 2002). In this study primary ESCs were treated with 8-Br-cAMP and MPA to induce a decidualised phenotype. Herein successful *in vitro* decidualisation, as measured by increase in IGFBP-1 mRNA and protein release ($p<0.05$ and $p<0.001$ respectively, n = 4 endometrial samples) has been demonstrated (Figures 3.2 – 3.3).

Previous studies have identified that endometrial tissues as well as endometrial stromal cells in culture express TGFβ type I and type II receptor mRNA and protein (Chegini, Zhao et al. 1994; Tang, Zhao et al. 1994; Dumont, O'Connor-McCourt et al. 1995; Piestrzeniewicz-Ulanska, Brys et al. 2002). The results in this study are in agreement with previous findings and demonstrated that cultured stromal cells (both non-decidualised and decidualised) express the mRNA for type I receptors (Figure 3.24 and 3.25). It has previously been reported that the level of TGFβ1 mRNA is higher in 1st trimester decidua as compared to endometrial tissue from the proliferative and secretory phases of the menstrual cycle (Kauma, Matt et al. 1990; Selick, Horowitz et al. 1994) and TGFβ type I receptors have been identified in term placenta (Dungy, Siddiqi et al. 1991; Mitchell and O'Connor-McCourt 1991; Mitchell, Fitz-Gibbon et al. 1992; Schilling and Yeh 2000). Initially it would appear, in this study, that decidualised cells have higher levels of TGFβ type I mRNA than non-decidualised cells (Figure 3.4) however with quantitative analysis there is no significant difference (Figure 3.5). The data from this study would suggest that TGFβ type I mRNA levels do not change in decidualised cells *in vitro*. This is in contrast to recent immunohistological findings (Kim, Park et al. 2005) which state that protein levels of both TGFβ type I and type II receptors is significantly upregulated in the stromal compartment within the secretory endometrium. The tissue sections used in that study (Kim, Park et al. 2005) were not classified into early, mid and late proliferative and secretory phases, so it is impossible to ascertain if the upregulation in the recptors was associated with decidualisation of the stroma, or just with increasing progesterone levels. To date, no data are presented to either

confirm or refute any alteration in receptor level with pregnancy. The presence of the receptors on the cells suggests that TGFβ1 can form the signalling receptor complex to mediate its biological effects locally not only in the culture system but also *in vivo*.

The initial objective was to identify whether TGFβ1 was responsible for the myofibroblast differentiation associated with the perivascular stromal cells. With this in mind, the ESCs were treated with TGFβ1, a known inducer of SMAα (Koumas, Smith et al. 2003). In this study, expression of SMAα did not significantly increase in non-decidualised or decidualised cells until 36 h ($p<0.001$ and $p<0.01$ respectively, n = 9 endometrial samples) and this increase was maintained over a period of 48 and 72 h of TGFβ1 treatment compared with controls ($p<0.001$, n = 9 endometrial samples) (Figures 3.7 and 3.8). This is in agreement with Grotendorst *et al* (Grotendorst, Rahmanie et al. 2004) who have also shown that fibroblast cells treated with TGFβ1 were SMAα positive with maximal SMAα protein expression observed at 36 h.

Prior to treatment with TGFβ1, we measured the correlation between endogenous TGFβ1 and endogenous SMAα expression in control ESC and found there to be a strong, positive, significant correlation between the two ($r=0.7459$, n = 9 endometrial samples) (Figure 3.9) suggesting that whilst both SMAα and TGFβ1 are present in the cells prior to any TGFβ1 treatment, further treatment with TGFβ1 can potentiate SMAα expression.

We report that TGFβ1 significantly inhibits mRNA and protein expression of PR in both non-decidualised and decidualised endometrial stromal cells in a time-dependent manner after an initial significant upregulation over a period of 2 h (Figures 3.10 -3.14). No explanation has been elucidated for the initial upregulation of PR at 2 h, but we have postulated that this could be attributed to the putative non-genomic PRs that have recently been identified (Losel and Wehling 2003; Zhu, Bond et al. 2003). The results presented herein demonstrated that this inhibition is not

attributable to TGFβ1 preventing PR gene transcription as presented in Figure 3.15. Several studies have reported that PR is under dual control of both oestradiol and progesterone acting at the oestrogen and progesterone response elements (Savouret, Bailly et al. 1991; Tseng and Zhu 1997; Tang, Mazella et al. 2002). We have demonstrated that our transient transfection of PRE and ERE into ESC was successful, as assessed by reporter gene assays (Figure 3.15). When treated with the appropriate steroid receptor-agonist, luciferase activity was increased demonstrating functionality of the response elements (Figure 3.15). Furthermore, when TGFβ1 was introduced into the culture it did not activate reporter activity and when introduced in combination with the steroid receptor-agonist it did not have any effect on reporter activity (Figure 3.15). In addition, TGFβ1 was without effect on specific steroid receptor-agonists binding to the ARE and GRE (which are identical to the PRE) (Figure 3.15). We have also demonstrated that ESCs contain endogenous TGFβ1 and neutralising this will negate any TGFβ1-induced effects, providing conclusive evidence that the downregulation of PR is TGFβ1-specific (Figure 3.20). This is the first evidence of TGFβ1 acting as a progesterone receptor modulator to inhibit mRNA and protein expression of PR.

Previous studies have reported that TGFβ1 inhibited expression of progesterone-induced genes e.g enkephalinase (Casey and MacDonald 1996) which acts to degrade bioactive peptides, and endothelin-1 (ET-1), a potent vasoconstrictor implicated in promotion of vasospasm in the spiral arterioles just prior to menses. It has been suggested that whilst TGFβ1 promotes selective progesterone-induced modifications it opposes others, implying that TGFβ1 acts as a PR-independent, gene specific anti-progestin though evidence to support such suggestions is scant. Further evidence to support a relationship between progesterone and TGFβ1 are provided in previous findings from Bruner *et al* (Bruner, Eisenberg et al. 1999), who report that TGFβ1 and progesterone are intimately involved in the prevention of experimental endometriosis in the nude mouse model. This may be due to impairment of MMP

regulation and had lead to the suggestion that TGFβ1 could be used for treatment of endometriosis (Dou, Williams et al. 1997).

In the studies presented herein, TGFβ1 upregulates protein expression of PIAS γ (Figure 3.14). This is in agreement with previous studies reporting that TGFβ1 induced expression of endogenous PIAS γ (Imoto, Sugiyama et al. 2003). PIAS γ is reported to inhibit SMAD transcriptional activity and other transcriptional responses. PIAS γ does not inhibit SMAD complex binding to DNA, but it represses SMAD transcriptional activity (Imoto, Sugiyama et al. 2003). Interestingly, other studies have elucidated that conditional overexpression of PIAS γ selectively inhibits a subset of endogenous TGF-beta-responsive genes, which includes PAI-1 further providing evidence for the negative-feedback that TGFβ1 has on its own production (Long, Matsuura et al. 2003). The story becomes more complex as recent studies have demonstrated that PIAS γ is complexed to the PR in human ESCs and that its ability to repress STAT1 signalling is dependent upon activation of PR in response to hormone binding (Zoumpoulidou, Jones et al. 2004). The same study reported that IFNγ and PIAS γ synergistically inhibited PR-dependent transcription, demonstrating that the progesterone and IFNγ signalling pathways engage in reciprocal transcriptional antagonism in human endometrium (Zoumpoulidou, Jones et al. 2004). The influx of uNK cells in the early secretory phase of the cycle coincides with an acute increase in endometrial IFNγ expression, a Th 1-type cytokine strongly implicated in effecting vascular remodelling necessary for embryo implantation and subsequent placenta formation (Ashkar, Di Santo et al. 2000; Ashkar and Croy 2001; Ashkar, Black et al. 2003; Croy, Esadeg et al. 2003). IFNγ inhibits the TGFβ-induced phosphorylation of SMAD3 and its attendant events, namely, the association of SMAD 3 with SMAD 4, the accumulation of SMAD 3 in the nucleus, and the activation of TGFβ-responsive genes. Acting through its associated protein tyrosine kinase, Jak1, and Stat1, IFNγ induces the expression of SMAD 7, an antagonistic SMAD, which prevents the interaction of SMAD 3 with the TGFβ receptors (Ulloa, Doody et al. 1999; Eickelberg, Pansky et al. 2001). A more recent study has elucidated that IFNγ interferes with TGFβ-signalling via direct interaction of Y-box-

binding protein (YB-1) with SMAD 3 to prevent subsequent translocation to the nucleus and TGFβ-induced gene transcription (Higashi, Inagaki et al. 2003). TGFβ1 may act to upregulate PIAS γ, which is complexed to PR, to interact with IFNγ, present in the late-secretory phase, to inhibit further production or activation of TGFβ1, hence limiting its own biological actions. Furthermore, in our findings PIAS γ protein expression is only modestly upregulated after 72 h, implying that its regulatory effects on TGFβ1 function are delayed (Figure 3.14). Recent evidence suggests that PIAS γ also represses other transcription factors, including the activated androgen receptor (AR), independent of its SUMO ligase activity (Gross, Yang et al. 2004).

Interestingly a recent study has provided evidence that DKK is up-regulated in decidualising endometrial stromal cells during the secretory phase of the menstrual cycle, suggesting regulation by progesterone (Giudice 2004). This finding was supported by data from *in vitro* culture of ESC, showing upregulation by progesterone of DKK mRNA synthesis and protein expression (Tulac, Overgaard et al. 2006). It was also reported that the response could be abrogated by the introduction of RU486 or progesterone withdrawal in long-term cultures, thereby providing evidence that DKK upregulation is mediated specifically by progesterone and independent of cAMP and oestradiol (Tulac, Overgaard et al. 2006). We are in agreement with findings from Tulac *et al* and have demonstrated significant DKK protein release with decidualisation in ESC ($p<0.001$, $n = 6$ endometrial samples) (Figures 3.19 and 3.21). Accumulating data would suggest a role for DKK in promoting cellular differentiation. It has been reported that Wnt-signalling in maturing osteoblasts needs to be downregulated to enable the formation of a mineralised bone matrix, and that this is, in part, due to DKK function (van der Horst, van der Werf et al. 2005). A recent study has reported that DKK, is secreted by human preadipocytes and promotes adipogenesis (Christodoulides, Laudes et al. 2006) further adding to the evidence that the normal Wnt-signalling pathway is involved in proliferation and that antagonism of the pathway by DKK promotes differentiation. The data herein demonstrate that TGFβ1 significantly downregulates

DKK mRNA expression in a biphasic manner ($p<0.001$, n=6 endometrial samples) (Figure 3.16) and downregulates protein release ($p<0.001$, n = 6 endometrial samples) (Figure 3.17) in non decidualised cells. The present studies also demonstrate that TGFβ1 significantly downregulates DKK mRNA expression in decidualised ESCs over a period of 24 h and 36 h, after which mRNA levels return to control ($p<0.05$, n= 6 endometrial samples) (Figure 3.18). Protein release was also significantly downregulated, but at a slower rate consistent with the expected mRNA and protein kinetics ($p<0.001$, n=6 endometrial samples) (Figure 3.19). This response is TGFβ1 specific as abolishing endogenous TGFβ1 prevents downregulation of DKK protein release (Figure 3.21). The present results show that TGFβ1 downregulates DKK mRNA before it downregulates PR mRNA. If DKK is indeed involved in decidualisation then perhaps this initial inhibition heralds the onset of TGFβ1-induced abrogation of the decidualised phenotype in the ESC.

SMAD proteins transduce signals from TGFβ binding to its serine/threonine kinase receptors to regulate its biological actions (Graff, Bansal et al. 1996; Zhang, Feng et al. 1996; Liu, Sun et al. 1997; Nakao, Roijer et al. 1997). The findings herein are in agreement with those of (Graff, Bansal et al. 1996; Zhang, Feng et al. 1996; Liu, Sun et al. 1997; Nakao, Roijer et al. 1997) who have reported that SMAD 2 and SMAD 3 are rapidly phosphorylated in response to TGFβ signalling (Figures 3.22 – 3.24). To determine if the TGFβ-induced responses we have observed are mediated via the SMAD-signalling pathway we attempted to selectively silence SMAD 4. Our silencing was successful as shown in Figures 3.25 and 3.26. However, it is impossible to determine whether the TGFβ1-induced downregulation of PR is SMAD dependent as the cells that had been transfected with the non-targeting siRNA demonstrated a significant reduction in the expression of PR mRNA (Figure 3.27). Some recent publications have focused on this "off-target gene regulation" of the non-targeting siRNA, and several groups are concentrating on designing an effective non-targeting siRNA devoid of any "off-targeting" properties (Jackson, Bartz et al. 2003; Birmingham, Anderson et al. 2006; Fedorov, Anderson et al. 2006).

To summarise, the studies performed in this chapter have demonstrated that TGFβ1 binds to its receptors in the ESC, both non-decidualised and decidualised, to promote myofibroblast differentiation, downregulation of PR at a transcript and protein level, induction of PIAS γ and inhibition of DKK, and induces phosphorylation of SMAD 2, required for propagating its effects via the SMAD-signalling pathway. These effects have potential consequences for regulating processes such as decidualisation, perhaps by inducing the Wnt-signalling pathway, and in the absence of pregnancy, menstruation.

Chapter 4

4 Impact of TGFβ1 on gene expression of decidualisation marker proteins

4.1 Introduction

4.1.1 Decidualisation

Decidualisation, the process by which progesterone acts on the oestrogen-primed endometrium to convert precursor stromal cells to decidual cells, epitomises the extensive sequential development of the human endometrium essential for successful implantation and continued pregnancy (Milligan, Cohen et al. 1995; Wilcox, Baird et al. 1999; King 2000; Lessey 2000). This decidualisation reaction is initiated in the perivascular stromal cells and under the continued influence of progesterone, spreads wave-like throughout the stromal region, occurring independently of the presence of a blastocyst, or with successful implantation, contributing to the formation of decidua in pregnancy (Bell 1990). Decidualisation is characterised by the transformation of the elongated fibroblast-like phenotype of endometrial stromal cells (ESC) to a larger, spherical decidual cell with intracellular structural rearrangements and concomittantly increasing stromal population. Studies *in vitro* on primary cultures of human endometrial stromal cells (ESCs) have revealed that this process is complex and likely to involve factors such as PGE_2 (Frank, Brar et al. 1994), relaxin (Tabanelli, Tang et al. 1992; Lane, Oxberry et al. 1994) and cAMP (Tang, Guller et al. 1993; Yee and Kennedy 1993; Gellersen, Kempf et al. 1994; Brar, Frank et al. 1997; Brosens, Hayashi et al. 1999; Mote, Balleine et al. 2000; Mak, Brosens et al. 2002) in addition to progesterone. The relative activities of the endometrial cells and their mediators may regulate the balance between tissue formation and breakdown.

4.1.2 Decidualised stromal cell functions

The decidualised stroma undertakes more extensive functions than a mere supportive structural role and a strong association exists between the degree of trophoblast invasion and the extent of decidualisation in species with a hemochorial placenta (Finn 1996). Not only does human trophoblast exhibit the greatest degree of trophoblast invasion seen in all species, but human endometrium undergoes the most extensive decidualisation reaction (Ramsey, Houston et al. 1976; Bell 1990). Decidualised stromal cells are temporally and spatially positioned to promote local homeostasis during implantation and counteract the threat of haemorrhage during trophoblast invasion (Lockwood, Krikun et al. 1999). Progesterone receptor knock-out mice fail to exhibit decidualisation and this is associated with implantation failure (Rider 2002). Trophoblast invasion in humans is significantly deeper in areas where the uterus is deficient in decidua caused by scar tissue (King and Loke 1999; Moffett and Loke 2006). Imbalances in implantation and placentation can result in abnormal placentation and the later development of pre-eclampsia (Loke and King 1997) highlighting the necessity for tight regulation of these events. Decidual cells are also thought to be important in preventing uterine bleeding in the peri-implantation phase of the cycle (Schatz, Krikun et al. 2001).

4.1.3 Decidualisation and menstruation

A previous study has argued that menstruation appears to only occur in species in which the decidualisation reaction in initiated spontaneously within the cycle in the absence of a blastocyst (Finn 1987). In the non-pregnant endometrium the interruption of the processes of decidualisation by steroid removal contributes to the process of menstruation. The concept that loss of decidual cell function promotes tissue breakdown mediated via loss of tissue breakdown suppressive properties or direct activation of tissue breakdown activity was proposed by Bell *et al* (Bell 1990). Although the classic "trigger" for the onset of menstruation is the withdrawal of progesterone, multiple cytokines and growth factors have been reported to play a role in this event e.g IL-1, IL-8 and PGs (Pickles 1967; Rampart, Van Damme et al. 1989;

Critchley, Kelly et al. 1994; Baird, Cameron et al. 1996; Singer, Marbaix et al. 1997; Milne, Critchley et al. 1999). Another potential candidate is transforming growth factor β1 (TGFβ1), which was the subject of studies described in chapter three. It is notable that several studies have reported a marked inhibitory effect of TGFβ1 on basal and stimulated PRL secretion, mRNA levels and *de novo* PRL synthesis in rat anterior pituitary cells (Herrlich, Kuhn et al. 1996; Telgmann, Maronde et al. 1997; Coya, Alvarez et al. 1999), indicating a potential inhibitory regulatory role for TGFβ1.

A better understanding of the local mechanisms involved in the regulation of endometrial events involved in menstruation is essential to understand the mechanisms involved in aberrant and abnormal bleeding associated with distressing menstrual disorders such as menhorragia (heavy menstrual bleeding) and dysmenhorrea (painful periods) and early pregnancy complications. In the current study we have therefore investigated the possibility that TGFβ1 may be involved in the initiation of menstruation by inhibiting expression and function of decidualisation products such as prolactin, IGFBP-1 and tissue factor.

4.1.4 Hypothesis

Although the studies in Chapter 3 have demonstrated that TGFβ1 is capable of inhibiting the mediators of decidualisation, PR and DKK, the effect of TGFβ1 on the classical decidualisation marker proteins; tissue factor (TF) (Lockwood, Krikun et al. 1994), IGFBP-1 (Irwin, Kirk et al. 1989) and dPRL (Maslar and Riddick 1979) is unknown. We hypothesise that TGFβ1 may suppress the production of the decidualisation marker proteins and therefore may play a role in the onset of menstruation.

4.1.5 AIMS

The studies described in this chapter were designed to determine the effect of TGFβ on gene expression of decidualisation marker proteins. All studies utilised primary endometrial stromal cells isolated from non-pregnant endometrium and 1st trimester decidua as described in section 2.1 and 4.2.1.2 respectively. Experiments in this chapter were designed to address the following questions:

1: Recent studies have identified TGFβ1 as an *in vitro* inhibitor of pituitary prolactin (Herrlich, Kuhn et al. 1996; Telgmann, Maronde et al. 1997; Coya, Alvarez et al. 1999) however, does TGFβ1 inhibit the expression of the decidualisation markers decidual prolactin (dPRL), IGFBP-1 and TF in ESCs, decidualised *in vitro*?

Furthermore, does neutralising endogenous TGFβ1 negate any TGFβ1-induced responses?

2: Does TGFβ1 interact with the dPRL promoter to inhibit decidualisation by preventing transcription of the prolactin gene?

3: Is the current well-characterised model of decidualisation the best *in vitro* model with which to evaluate regulation of decidualisation markers?

4: Does TGFβ1 inhibit the expression of the decidualisation markers prolactin, IGFBP-1 and TF in decidual stromal cells (DSC) obtained from 1st trimester decidua?

5: Can selectively silencing SMAD 4, discussed in section 3.3.9, prevent any TGFβ1-induced responses?

4.2 Methods

4.2.1 Human uterine tissue collection

4.2.1.1 Endometrium from non pregnant women

Endometrial (n = 18) samples were collected as detailed in section 2.1.1 (Table 2.1a). Endometrial biopsies utilised in this chapter are detailed in Table 4.1. All endometrial samples were processed to isolate the stromal compartment from the glands, as described in section 2.2. Biopsies were collected with an endometrial suction curette (Pipelle, Laboratoire CCD) or alternatively, full thickness endometrial samples were obtained. Such tissue samples included superficial and basal endometrium plus the endometrial-myometrial junction. All women described regular menstrual cycles (25-35 days) and had not received any form of exogenous hormonal treatment in the 3 months preceding biopsy. Biopsies were dated according to the patient's reported last menstrual period (LMP) and only included for analysis if the histological dating according to published criteria (Noyes, Hertig et al. 1950) and circulating sex steroid concentrations (E_2 and P at time of endometrial biopsy) were consistent with the LMP. Serum was separated (by centrifugation at 800 g for 7 min) from venous blood samples collected at the time of biopsy and oestradiol (E_2) and progesterone (P) concentrations were measured by radioimmunoassay.

4.2.1.2 Tissue Collection and processing of human decidual tissue

Decidual (n = 11) samples were collected as detailed in section 2.1.1 (Table 2.1b). Decidual biopsies utilised in this chapter are detailed in Table 4.2. All decidual samples were processed to isolate the stromal compartment, depleted of $CD56^{bright}$ uterine natural killer cells (uNKs), from the glands. Decidual tissue was finely minced into 1 cm^3 sections using surgical blades (Swann) and residual blood clots were removed. Approximately 10 g of the minced tissue was placed in 20 ml of RPMI with 10 % FCS, 4 ml of collagenase (2 mg/ml) and 0.5 ml of DNAse (0.1

mg/ml; Sigma) for 1 h 20 min on a roller at 37 °C. After digestion, 30 ml of RPMI 10 % FCS was added and the mixture was left to stand for 5 min to allow sedimentation. The supernatant was decanted by aspiration and passed sequentially through 73 μm and 40 μm filters (VWR). The filtrate was centrifuged at 400 g for 5 min and the resulting cell pellet was resuspended in 15 ml of PBS supplemented with 2 % FCS and 0.1 % NaN_3 and subsequently overlaid onto 15 ml of Lymphoprep™ (Axis-Shield, Oslo, Norway) before further centrifugation at 710 g for 20 min with no brake. The cells at the interface were collected; these consisted of 60 to 80 % uNKs, 5 – 15 % $CD14^+$ macrophages and 10 to 20 % T cells plus some stromal and epithelial cells. The cells were washed in 20 ml of RPMI 10% FCS and pelleted by centrifugation at 710 g for 5 min. $CD56^{bright}$ uNKs were further purified by positive selection using CD56 antibody-coated magnetic Microbeads and a magnet assisted cell separation (MACS®) (Miltenyi Biotec Ltd, Surrey, UK) protocol, detailed in section 5.2.1. After removing the uNKs from the cell preparation the remaining cells were transferred to a 75 cm^3 cell culture flask in 10 ml of RPMI 10 FCS and incubated at 37°C in a humidified atmosphere of 5 % CO_2. After 24 hours the media was changed and the non-adherent cells discarded. The adherent cells, the decidual stromal cells (DSC), were allowed to attain confluence and used for experimentation at 1^{st} passage.

Patient	Age	Cycle stage	Blood E2 (pg/ml)	Blood P4 (ng/ml)	Ethics No
1	37	6	215	1.8	17
2	33	11	373	1.8	17
3	39	13	964	3.9	17
4	37	7	234	1.7	17
5	33	6	255	2.8	17
6	44	13	359	1.8	17
7	39	10	835	10.14	17
8	42	14	478	23.66	17
9	41	9	1002	13.68	17
10	37	10	N/A	N/A	17
11	38	10	353	63	17
12	47	11	1796	2.32	17
13	30	4	242	6.15	40
14	43	8	581	68.94	17
15	32	12	1651	4.04	17
16	40	12	667.03	10.86	17
17	42	8	208.52	0.88	17
18	35	9	278.52	2.45	17

Table 4.1: Details of biopsies used in the studies presented in Chapter 4.

Patient	Age	D.O.C	Gestation (Wks+day)	Ethics No
1	21	28/9/05	8	05/s1104/12
2	30	28/9/05	9+2	05/s1104/12
3	18	29/9/05	8+5	05/s1104/12
4	20	4/10/05	9.3	05/s1104/12
5	24	4/10/05	8.3	05/s1104/12
6	30	12/10/05	9.3	05/s1104/12
7	24	12/10/05	9.6	05/s1104/12
8	27	26/10/05	11+1	05/s1104/12
9	28	28/11/05	10+2	05/s1104/12
10	23	29/11/05	11	05/s1104/12
11	24	2/12/05	10+3	05/s1104/12

Table 4.2: Details of 1st trimester decidual biopsies used in the studies presented in Chapter 4. D.O.C. = Date of Collection.

4.2.2 *In vitro* primary cell culture experiments

ESCs were maintained at 37°C in 5 % (v/v) CO_2 in RPMI 1640 medium (Sigma) supplemented with 10 % FCS (Mycoplex), penicillin (50 μg/ml; Sigma), streptomycin (50 μg/ml; Sigma) and gentamycin (5 μg/ml; Sigma). Passaging of the cells was performed every 3-4 days by trypsinisation (section 2.2). The cells were seeded in 6-well plates at a concentration of 2.5 x 10^5 cells/ml and allowed to adhere and attain 90 % confluence: 24 h prior to treatment the media was changed to 2 % FCS RPMI. Decidualisation of the cells was induced with decidualising medium (DM) for 6 days, thereafter, the cells were treated with 2 % FCS RPMI 1640 and DM containing TGFβ1. Tables 4.3-4.6 and 4.8-4.9 detail the treatment regimes utilised. Cells were maintained under these conditions for up to 72 h. Each treatment was duplicated and each experiment was repeated 3-9 times with separate endometrial biopsies.

Experiment 4.1: Does TGFβ1 interfere with the expression of dPRL in ESC and DSC, decidualised *in vitro*?

Treatment	Concentration	Incubation period
Control	N/A	2, 12, 24, 36, 48, 72 h
MPA +8-Br-cAMP	1 μM + 250 μM	2, 12, 24, 36, 48, 72 h
MPA+8-Br-cAMP +TGFβ1	1 μM + 250 μM + 10 ng/ml	2, 12, 24, 36, 48, 72 h

Table 4.3: Experimental regime for experiment 4.1

Experiment 4.2: Does TGFβ1 interfere with the expression of IGFBP-1 in ESC and DSC, decidualised *in vitro*?		
Treatment	Concentration	Incubation period
Control	N/A	2, 12, 24, 36, 48, 72 h
MPA +8-Br-cAMP	1 μM + 250 μM	2, 12, 24, 36, 48, 72 h
MPA+8-Br-cAMP +TGFβ1	1 μM + 250 μM + 10 ng/ml	2, 12, 24, 36, 48, 72 h

Table 4.4: Experimental regime for experiment 4.2

Experiment 4.3: Does TGFβ1 interfere with the expression of TF in ESC and DSC, decidualised *in vitro*?		
Treatment	Concentration	Incubation period
Control	N/A	2, 12, 24, 36, 48, 72 h
MPA +8-Br-cAMP	1 μM + 250 μM	2, 12, 24, 36, 48, 72 h
MPA+8-Br-cAMP +TGFβ1	1 μM + 250 μM + 10 ng/ml	2, 12, 24, 36, 48, 72 h

Table 4.5: Experimental regime for experiment 4.3

Experiment 4.4: Does neutralising endogenous TGFβ1 abrogate TGFβ1-induced responses?		
Treatment	Concentration	Incubation period
Control	N/A	72 h
MPA +8-Br-cAMP	1 μM + 250 μM	72 h
MPA+8-Br-cAMP +TGFβ1	1 μM + 250 μM + 10 ng/ml	72 h
MPA+8-Br-cAMP + Anti-TGFβ1	1 μM + 250 μM + 2 ng/ml	72 h
Mouse IgG1		72 h

Table 4.6: Experimental regime for experiment 4.4

4.2.3 RNA extraction

Total RNA was extracted from the ESC and DSC cells as described in section 2.3.1 and cDNA prepared from the experiments detailed in Tables 4.3-4.6 and 4.9 as described in section 2.3.2.

4.2.4 Q-RT-PCR

The relative levels of expression of IGFBP-1, dPRL and TF in ESC and DSC cells, both control and treated cells (Tables 4.3 – 4.6 and 4.9), were determined using quantitative RT-PCR (TaqMan) using random primed cDNA as detailed in section 2.4.2.

Results were analysed as in section 2.4.2. Primer pairs and probes specific to each gene are given in Table 4.7.

Amplicon	Accession Number	Forward Primer	Reverse Primer	Probe
IGFBP-1	M59316	CACAGGAG ACATCAGG AGAAGAAA	ACACTGTCT GCTGTGATA AAATCCAT	TTCCAAATTTT ACCTGCCAAA CTGCAACAA
dPRL	NM_000948	GCCCCGGA GGCTATCC TA	TCAGCTCCA TGCCCTCTA GAA	CCAAAGCTGT AGAGATTCAG GAGCAAACCA
Tissue Factor	NM_001993	CACCGACG AGCTTGTG AAGGA	CCCTGCCGG GTAGGAGA A	TGAAGCAGAC GTACTTGGCA CGGGT

Table 4.7: Primer and probe sequences

4.2.3 Transient transfection

Transient transfections were performed using the reporter vectors: dPRL-3000/luc3, dPRL-913/luc3, dPRL-601/luc3, dPRL-332/luc3, and dPRL-32/luc3 carrying 3000, 913, 601, 332 and 32 bp, respectively, of 5'-flanking DNA to the decidual-specific promoter of the hPRL gene, linked to luciferase (section 2.5.1) A β-galactosidase control vector, (PCH110, Pharmacia), was co-transfected in all cases and used to measure β-galactosidase to control for transfection efficiency (section 2.5.4). Transient transfections were performed using the calcium chloride method as described in section 2.5.2. All transfections were performed in triplicate, using DCC media, a 1:1 mixture of DMEM and Ham's F12 containing 5% FCS that had been depleted of steroids by treatment with dextran-coated charcoal, 100 U/ml penicillin, and 100 μg/ml streptomycin, and supplemented with 10^{-9} M 17ß-oestradiol and 1 μg/ml insulin (section 2.5.2).

Six hours after transfection the media was replaced with 2% FCS DCC. The experiments were performed as detailed in Table 4.8. The cells were harvested 48 h after treatment for analysis with a luciferase assay (section 2.5.3). Tranfection efficiency was analysed with a β-galactosidase assay (section 2.5.4). The samples were "normalised" by dividing each sample's luciferase reading with the corresponding β-galactosidase reading. This numerical reading was used as a comparison against other samples.

Experiment 4.5: Does TGFβ1 repress the prolactin promoter transactivation potential?		
Treatment	Concentration	Incubation period
MPA +8-Br-cAMP	1 μM + 250 μM	72 h
MPA+8-Br-cAMP +TGFβ1	1 μM + 250 μM + 10 ng/ml	72 h

Table 4.8: Experimental regime for experiment 4.5

4.2.4 siRNA for SMAD 4

ESCs were transiently transfected using the calcium phosphate precipitation method, as previously described in section 2.5.1, with 50 nM of the siRNA reagents (Dharmacon) detailed in Table 4.9. The RNA and protein content was extracted 72 h later as described in sections 2.3.1 and 2.6.1 respectively.

Experiment 4.6: Does interfering with the SMAD signalling pathway inhibit TGFβ1-induced responses?		
Treatment	Concentration	Incubation period
Mock transfection	N/A	72 h
Control siRNA (SiGENOME SMARTpool) MPA +8-Br-cAMP	1 μM + 250 μM	72 h
SMAD 4 siRNA (SiGENOME SMARTpool) MPA +8-Br-cAMP	1 μM + 250 μM	72 h
Control siRNA (SiGENOME SMARTpool) MPA +8-Br-cAMP + TGFβ1	1 μM + 250 μM 10 ng/ml	72 h
SMAD 4 siRNA (SiGENOME SMARTpool) MPA+8-Br-cAMP +TGFβ1	1 μM + 250 μM 10 ng/ml	72 h

Table 4.9: Experimental regime for experiment 4.5

4.2.5 Time-resolved fluoroimmunoassay of prolactin in culture supernatants

Time-resolved fluoroimmunoassay of prolactin was conducted as described in section 2.6.8.

4.2.6 Slide preparation and immunohistochemical staining of cytokeratin

Slides were prepared as described in section 2.6.9.1 and immunohistochemical staining conducted as detailed in section 2.6.9. Briefly, tissue sections were incubated with mouse monoclonal anti-cytokeratin antibody (Dako UK Ltd) at a 1:100 diluition in blocking serum (section 2.6.9.5). After incubation with the primary antibody the slides were washed three times with TBS for 5 min each, and incubated with the secondary antibody, goat anti mouse biotinylated (Dako UK Ltd) at a diltution of 1:500 in the blocking serum. Immunolocalised proteins were detected with DAB.

4.2.7 Viable Cell Counts Using Trypan Blue

Trypan Blue is a vital dye which does not interact with the cell unless the membrane is damaged. Therefore, all the cells that exclude the dye are viable.

After trypsinisation (section 2.2), the cells were centrifuged at 400 g for 3 min to pellet the cells. Thereafter, the cells were resuspended in RPMI at a concentration of 2 x 10^5 cells per ml. 0.1 ml of 0.4 % Trypan Blue Stain (Sigma) was added to the cells and the solution was vortexed to mix thoroughly. After 5 min at room temperature the cell preparation was mixed again and 10 µl was added to a cell counting chamber (SLS, UK). Using a light microsope (Leica), the cells were observed to determine the number of viable cells.

4.2.8 Statistical Analysis

Prior to statistical analysis, all data were tested for Gaussian distribution. Where appropriate, values were presented as means ± S.E.M. Significant difference of the Q-RT-PCR, ELISA and transfection data was determined by repeated measures analysis of variance (ANOVA) using the computer package Prism 4.0. Significant differences were assigned using Kruskal-Wallis post hoc test. The criterion for significance for all tests was set at $p< 0.05$.

4.3 Results

4.3.1 TGFβ1 downregulates the production of decidualisation products

To determine the role of TGFβ1 in mediating decidualisation in the endometrium, ESC were cultured, decidualised *in vitro* for 6 d and then further treated with decidualising medium in the presence or absence of TGFβ1 (10 ng/ml) for 72 h. Quantitative RT-PCR analysis revealed that TGFβ1 significantly downregulated the expression of IGFBP-1 mRNA in a time-dependent manner with a 50 % decrease observed at 48 h (Figure 4.1) ($p<0.01$, n = 8 endometrial samples) and 72 h (Figure 4.1) ($p<0.001$, n = 8 endometrial samples) in ESC, decidualised *in vitro*, as compared to unstimulated, time-matched controls. Interestingly, it would appear that IGFBP-1 mRNA was potentiated at the 12 h time point; however this was not significant and may be attributed to the individual variability between samples. This significant decrease in IGFBP-1 mRNA was also observed in the amount of IGFBP-1 protein release as detected with a two-site sandwich ELISA (Figure 4.2). Interestingly, significant inhibition of protein release was seen after only 2 h of TGFβ1 treatment and did not return to control levels throughout the duration of the experiment, with a significant decrease observed at 12 h, 24 h, 48 h and 72 h (Figure 4.2) (All $p<0.001$, n = 6 endometrial samples). TGFβ1-induced inhibition of dPRL mRNA and protein release mirrored that of IGFBP-1, with significant inhibition of mRNA levels observed at 48 h ($p<0.001$, n = 6 endometrial samples) and 72 h ($p<0.001$, n = 6 endometrial samples), as measured by Q-RT-PCR (Figure 4.3). Protein release was significantly suppressed after only 2 h of TGFβ1 treatment ($p<0.05$, n = 6 endometrial samples) (Figure 4.4). TGFβ1 consistently evoked significant inhibition of dPRL protein release at all time points; 12 h ($p<0.01$, n = 6 endometrial samples) 24 h ($p<0.05$, n = 6 endometrial samples) 48 h ($p<0.01$, n = 6 endometrial samples) 72 h ($p<0.01$, n = 6 endometrial samples), as measured by time-resolved fluoroimmunoassay (Figure 4.4). In addition, TGFβ1-induced inhibition of tissue factor mRNA, with significant suppression of mRNA levels observed at 24 h

($p<0.05$, n = 6 endometrial samples), 48 h ($p<0.001$, n = 6 endometrial samples) and 72 h ($p<0.001$, n = 6 endometrial samples), as measured by Q-RT-PCR (Figure 4.5).

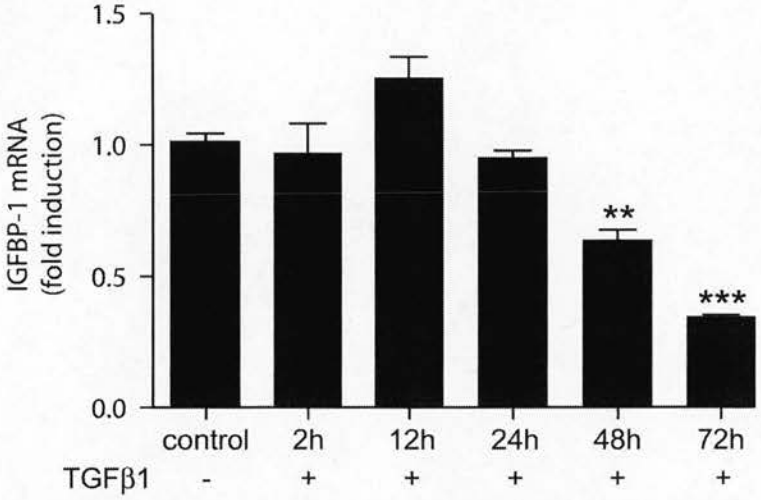


Figure 4.1: TGFβ1 inhibits expression IGFBP-1 mRNA expression. Cells were decidualised *in vitro* and treated in the presence or absence of TGFβ1 for a maximum of 72 h. TGFβ1 reduces expression of mRNA IGFBP-1 in a time dependent manner measured by Q-RT-PCR. 48 h, $p<0.01$, 72 h, $p<0.001$ n = 6 endometrial samples.

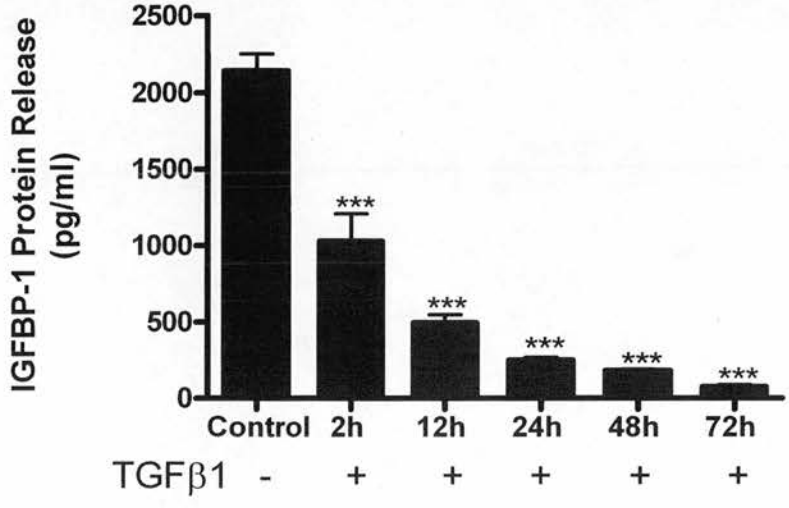


Figure 4.2: TGFβ1 inhibits IGFBP-1 protein release, measured by ELISA. Cells were decidualised *in vitro* and treated in the presence or absence of TGFβ1 for a maximum of 72 h. TGFβ1 reduces protein release of IGFBP-1 after only 2 h of treatment ($p<0.001$, n = 6 endometrial samples). This continues in a time-dependent manner (all time points $p<0.001$).

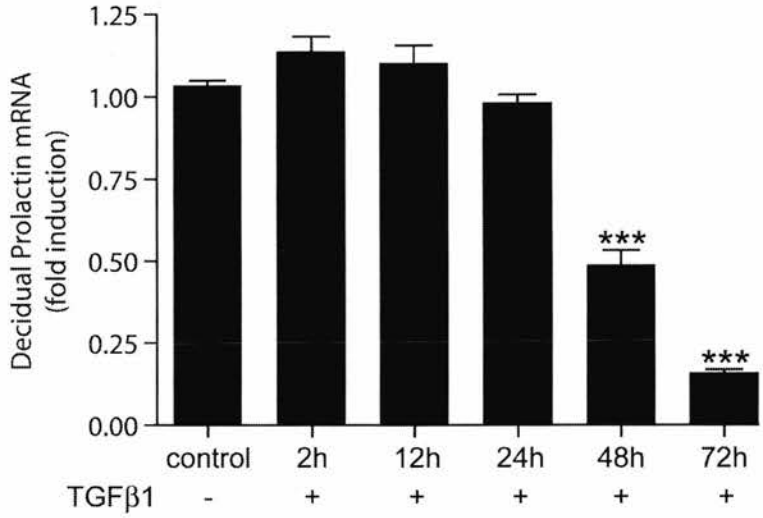


Figure 4.3: TGFβ1 inhibits expression of PRL mRNA expression. Cells were decidualised *in vitro* and treated in the presence or absence of TGFβ1 for a maximum of 72 h. TGFβ1 reduces expression of mRNA PRL in a time dependent manner; 48 h ($p<0.01$, n = 6 endometrial samples), 72 h ($p<0.001$, n = 6 endometrial samples).

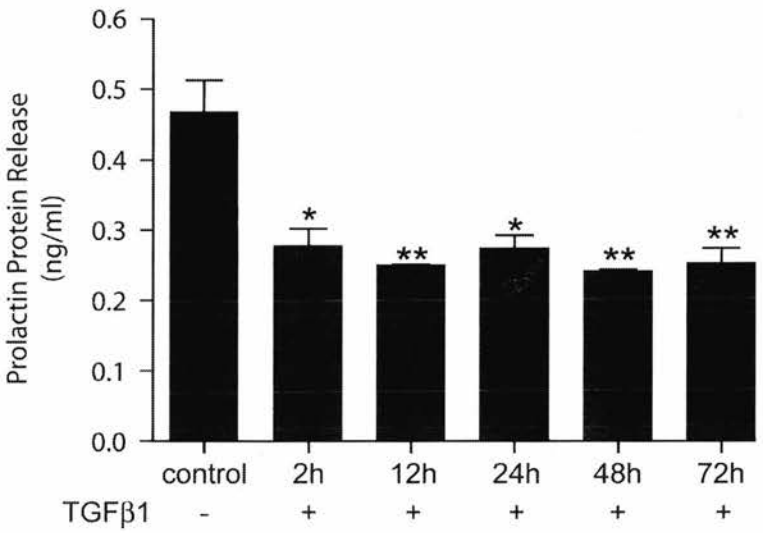


Figure 4.4: TGFβ1 inhibits prolactin protein release measured by time-resolved fluoroimmunoassay. Cells were decidualised *in vitro* and treated in the presence or absence of TGFβ1 for a maximum of 72 h. TGFβ1 significantly reduces protein release of prolactin in a time dependent manner (72 h, $p<0.01$) (n=6 endometrial samples).

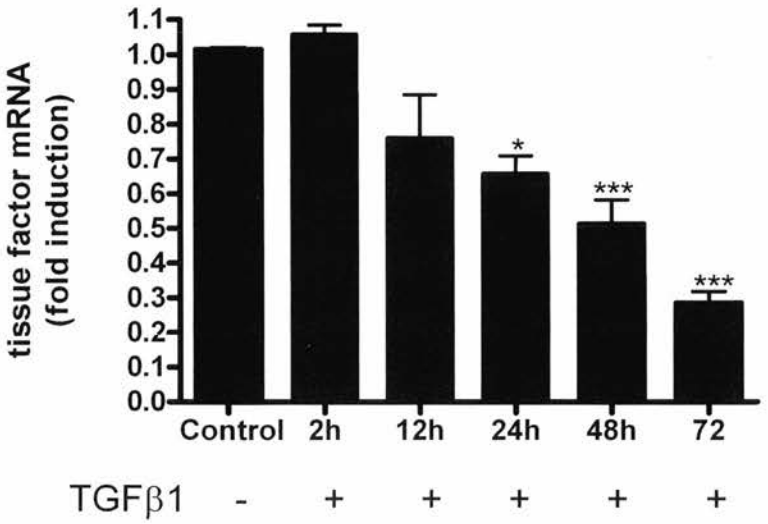


Figure 4.5: TGFβ1 inhibits expression of tissue factor mRNA as measured by Q-RT-PCR. Cells were decidualised *in vitro* and treated in the presence or absence of TGFβ1 for a maximum of 72 h. TGFβ1 reduces expression of tissue factor mRNA in a time dependent manner; 24 h ($p<0.05$, n = 8 endometrial samples), 48 h ($p<0.001$, n = 6 endometrial samples), 72 h ($p<0.001$, n = 8 endometrial samples).

4.3.2 TGFβ 1 represses the prolactin promoter transactivation potential

In order to identify if TGFβ1 could interact with the dPRL promoter to inhibit decidualisation, primary cultures of undifferentiated ESC and decidualising cells were transiently transfected with the dPRL. The results are presented in Figure 4.6: TGFβ1 inhibited promoter activity (approximately 50% inhibition), in all constructs containing the PRE half site. ~50 % repression was still apparent with a promoter construct containing 332 bp upstream of the decidual PRL transcription start site. This construct contains the critical decidualisation region (-270/-332) that confers the cAMP response as well as containing a PRE half site. No repression was seen with the promoter construct containing 32 basepairs upstream of the decidual PRL transcription start site (PRE negative).

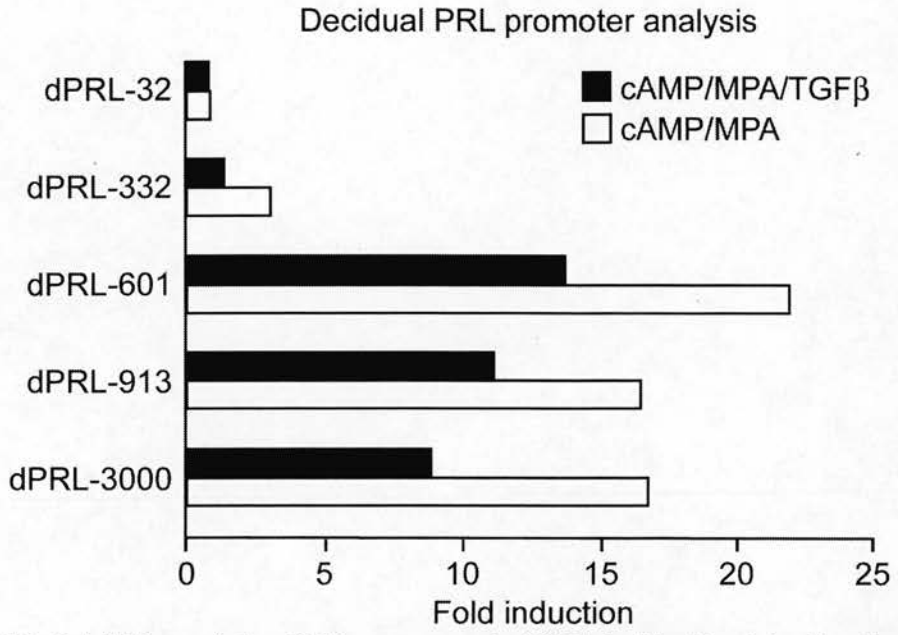


Figure 4.6: Inhibition of the dPRL promoter in ESC decidualised *in vitro* by TGFβ1 in a transient transfection assay. ESCs were transfected with a decidual PRL promoter containing 3000 kb of DNA flanking the decidual-specific start site (dPRL-3000/luc), or truncated vectors containing the critical decidualisation region, -332 bp to -270 bp, relative to the transcription site thereof. This site contains half site PRE's (Christian, Pohnke et al. 2002). TGFβ1 inhibits promoter activity of all constructs by approximately 50 %. Compare white and black bars. NB. No repression was seen with the promoter construct containing only 32 basepairs upstream of the decidual start site (PRE negative).

4.3.3 Anti-TGFβ1 neutralising antibody negates TGFβ1 inhibition of decidualisation genes

In an attempt to elucidate if blocking endogenous TGFβ1 action would negate the TGFβ1-induced responses, ESC were cultured in the presence and absence of decidualising medium for six days and then further cultured with decidualising medium to maintain the decidualised phenotype in the presence or absence of TGFβ1 (10 ng/ml) or anti-TGFβ1 antibody (1 μg/ml) for a period of 72 h. As previously seen, TGFβ1 significantly downregulates mRNA expression of dPRL (Figure 4.7. graph A) ($p<0.05$, n = 6 endometrial samples) and IGFBP-1 (Figure 4.7. graph B) ($p<0.001$, n = 6 endometrial samples). Treatment with anti-TGFβ1 abolishes this

response and significantly potentiated expression of IGFBP-1 mRNA in comparison with ESCs treated with decidualising mix alone (Figure 4.7. graph B) ($p<0.001$, n = 6 endometrial samples). Treatment with the isotype control, mouse IgG1, evoked no response. In every sample, anti-TGFβ1 antibody increased mRNA expression of dPRL; however the individual variability between samples prevented any statistically significant change between treatments (Figure 4.7. graph A).

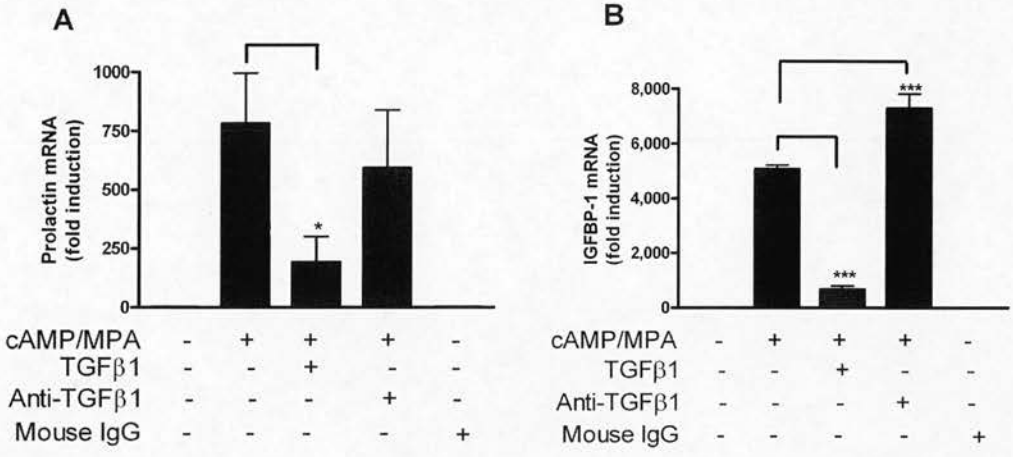


Figure 4.7: Anti-TGFβ1 antibody neutralises endogenous TGFβ1 and potentiates the decidualisation process. Cultured ESCs were decidualised *in vitro* +/- TGFβ1-treatment. To confirm the specificity of the TGFβ1 response anti-TGFβ1 (1 μg/ml) or mouse IgG control was added, for 72 h. A: TGFβ1 inhibits expression of decidual prolactin ($p<0.05$, n = 6 endometrial samples), whilst anti-TGFβ1 increases expression of decidual prolactin (n = 6 endometrial samples). B: TGFβ1 reduces expression of IGFBP-1 mRNA ($p<0.001$, n = 6 endometrial samples), whilst anti-TGFβ1 antibody increases IGFBP-1 ($p<0.001$, n = 6 endometrial samples).

4.3.4 Is the current well-characterised model of decidualisation the best *in vitro* model with which to evaluate regulation of decidualisation markers?

4.3.4.1 Characterisation of decidual stromal cells (DSC)

Before embarking on a series of experiments to determine if using stromal cells derived from 1st trimester decidua is a better *in vitro* model with which to evaluate

regulation of decidualisation markers than the present model, the presence or otherwise of trophoblast cells within the decidual tissue had to be confirmed, to ensure decidua used in the experiments were distal from the implantation site and free from trophoblast contamination. Figure 4.8 represents cytokeratin staining in decidua parietalis, panel I, and decidua basalis containing trophoblast villi, panel II. All decidual biopsies were stained for cytokeratin and proved to be negative from trophoblast contamination.

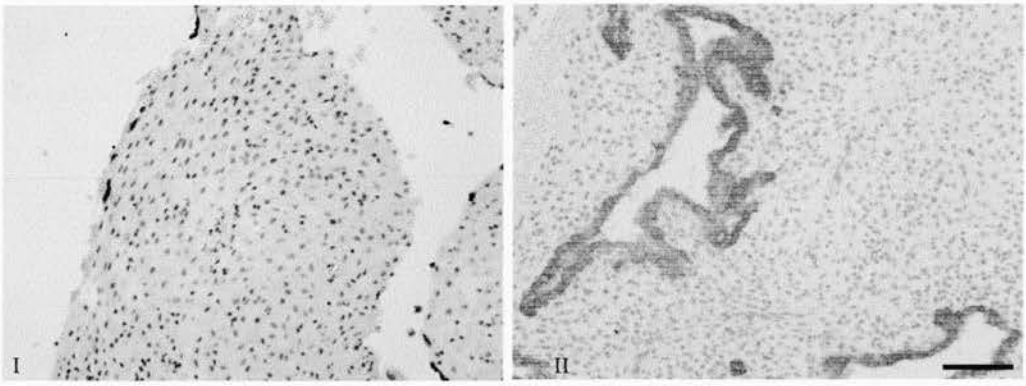


Figure 4.8: Cytokeratin staining in decidual biopsies. Panel I depicts a tissue section from a decidua parietalis biopsy. Panel II shows a tissue section from decidua basalis. Scale bars represent 100μm.

4.3.4.2 DSC extracted from decidua parietalis de-differentiate morphologically and release reduced concentrations of IGFBP-1

In order to address whether decidualisation is a terminal differentiation or if cells, exposed to decidualisation stimuli *in vivo*, can de-differentiate, DSC were cultured in the absence of any decidualisation stimuli for 14 d and the IGFBP-1 protein release measured at 3 d, 9 d and 14 d of culture. Results are presented in Figure 4.9; in each sample long-term culture (9 d) in the absence of decidualising stimuli demonstrated a significant reduction in IGFBP-1 protein release ($p<0.001$, n = 11 decidual samples) (Figure 4.9, Panel A), but after 9 d of culture DSCs still release sufficient levels of protein to be characterised as decidualised i.e. releasing >500 pg/ml/$5x10^5$ cells IGFBP-1. After 14 d of culture, without decidualising stimuli, IGFBP-1 protein

levels were significantly reduced 5-fold ($p<0.001$, n= 11 decidual samples) (Figure 4.9, Panel A), however approximately 3500 pg/ml/5x10^5 cells IGFBP-1 was still being released by the cells. Samples 8 - 11 were over 10 wks gestation at time of collection. These samples released significantly greater levels of IGFBP-1 protein than those samples from < 10 wks gestation ($p<0.001$) (Figure 4.9, Panel B) and still released approximately 10000 pg/ml/5x10^5 cells IGFBP-1 after 14 d in culture in the absence of decidualising stimuli ($p<0.001$) (Figure 4.9, Panel B). Morphologically the DSCs did appear to become more "fibroblast-like" over time, with an apparent decrease observed in the spherical shape associated with decidualised cells (Figure 4.10). In addition, the cells remained healthy and the reduction in IGFBP-1 release was not associated with cell death as determined by Trypan blue staining and cell counts (Figure 4.11).

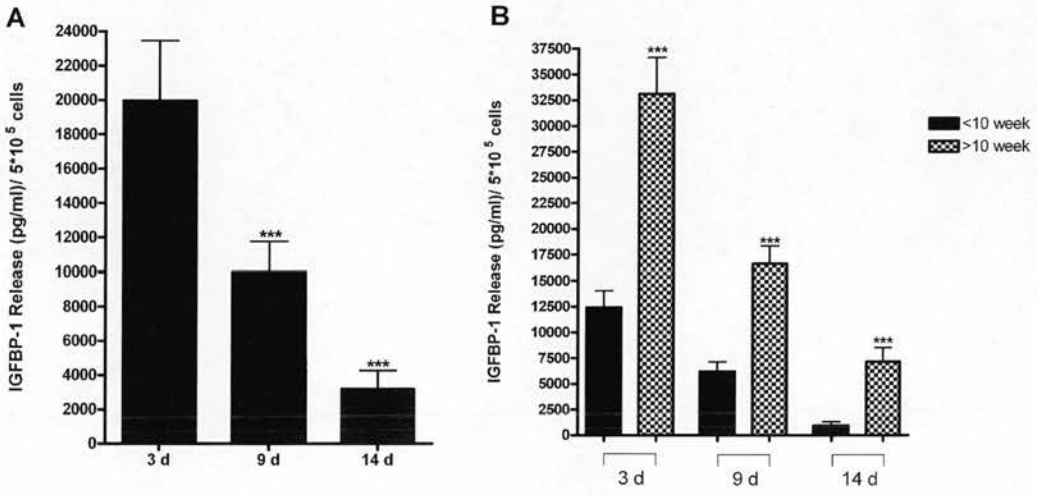


Figure 4.9: DSCs de-differentiate in culture. DSCs were cultured in the absence of decidualising stimuli for 14 d. Panel A; IGFBP-1 protein release was abrogated in a time-dependant manner after 9 d ($p<0.001$, n = 11 decidual samples). Continual suppression was observed after 14 d ($p<0.001$, n =11 decidual samples). Panel B; black bars denote samples < 10 wks gestation, chequered bars denote samples > 10 wks gestation. Samples < 10 wks release significantly less IGFBP-1 and are seen to ($p<0.001$) but samples > 10 wks gestation are still releasing ~ 10000 pg/ml/5x10^5 cells IGFBP-1 after 14 d in culture without decidualising stimuli. n = 7 decidual samples < 10 wks gestation, n = 4 decidual samples > 10 wks gestation.

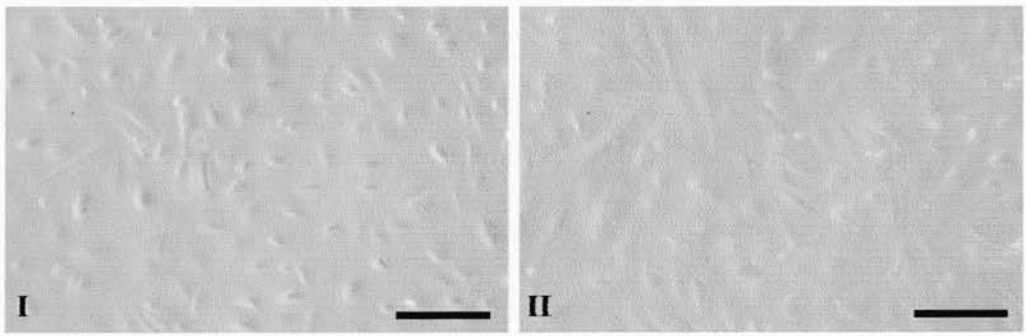


Figure 4.10: Photograph of DSC morphology in the absence of decidualising stimuli after 3 d (Panel I) and 14 d (Panel II) in culture. Scale bars represent 100μm.

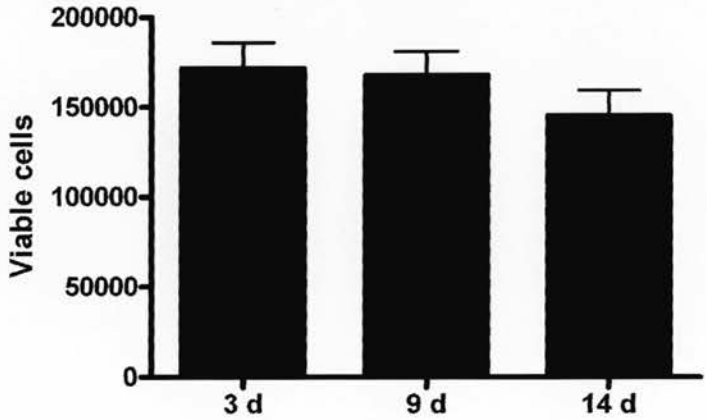


Figure 4.11: DSCs are still viable after 14 d in culture in the absence of decidualising stimuli (n = 6 decidual samples).

4.3.4.3 DSC decidualise faster than ESC

In order to elucidate if DSC re-decidualised faster than ESC, stromal cells from non-pregnant endometrium and the 7 samples obtained from decidua < 10 weeks gestation were cultured in the presence or absence of 8-Br-cAMP (0.5 mM) +/- MPA (1 μM) for up to 72 h. Decidualisation markers, dPRL and IGFBP-1, were measured by Q-RT-PCR and ELISA.

4.3.4.3.1 Prolactin

Decidual prolactin mRNA expression is significantly potentiated in DSC, re-decidualised *in vitro* with MPA and cAMP, after only 24 h of treatment (Figure 4.12) ($p<0.001$, n = 7 decidual and 7 endometrial) as compared with ESC treated identically. A significant difference in dPRL mRNA is also observed at 48 h (Figure 4.12) ($p<0.001$, n = 7 decidual and 7 endometrial biopsies) and 72 h (Figure 4.12) ($p<0.01$, n = 7 decidual and 7 endometrial biopsies) between DSC and ESC.

4.3.4.3.2 IGFBP-1

In contrast to dPRL findings, IGFBP-1 mRNA expression was significantly potentiated in ESC compared to DSC after both were treated with MPA alone (Figure 4.13) ($p<0.001$, n = 7 decidual and 7 endometrial biopsies) or MPA + cAMP for 12 h (Figure 4.13) ($p<0.001$, n = 7 decidual and 7 endometrial biopsies). However, after 48 h of treatment the opposite was observed in that IGFBP-1 mRNA expression was significantly potentiated in DSC compared to ESC after both were treated with MPA alone (Figure 4.13) ($p<0.001$, n = 7 decidual and 7 endometrial biopsies) or MPA + cAMP (Figure 4.13) ($p<0.001$, n = 7 decidual and 7 endometrial biopsies). No significant differences were observed at other time points. IGFBP-1 protein release in DSC compared with ESC after treatment with decidualising stimuli further supports the argument that DSCs re-decidualise faster. After only 2 h of treatment with MPA + cAMP IGFBP-1 release is significantly upregulated in DSC as compared to identically-treated ESC (Figure 4.14) ($p<0.001$, n = 7 decidual and 7 endometrial biopsies). The same is true for 24 h of treatment with MPA + cAMP (Figure 4.14) ($p<0.05$, n = 7 decidual and 7 endometrial biopsies). After 72 h of treatment IGFBP-1 protein expression was significantly potentiated in DSC compared to ESC after both were treated with MPA alone (Figure 4.14) ($p<0.001$, n = 7 decidual and 7 endometrial biopsies) or MPA + cAMP (Figure 4.14) ($p<0.05$, n = 7 decidual and 7 endometrial biopsies). No significant differences were observed at other time points.

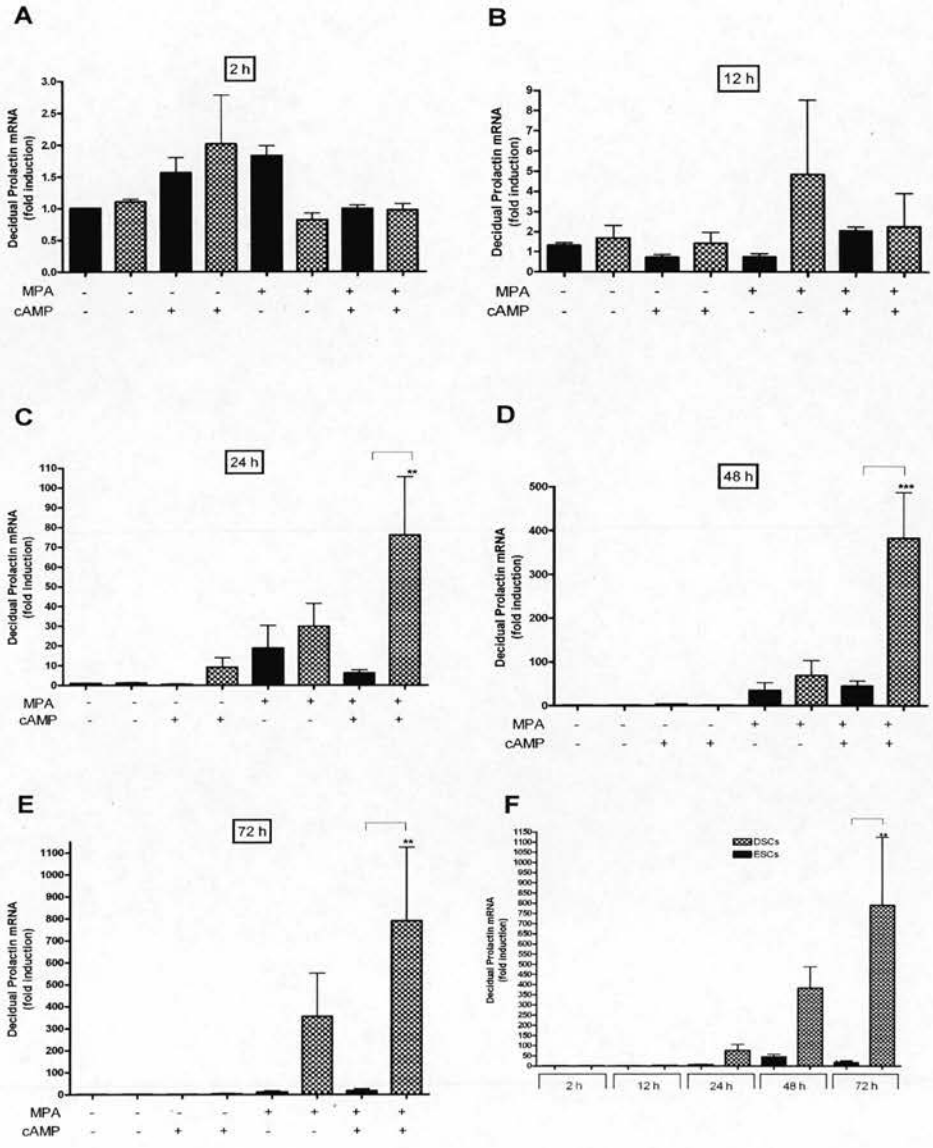


Figure 4.12: dPRL mRNA expression in DSC and ESC. DSC and ESC were cultured in the presence or absence of decidualising stimuli, cAMP (250 μM) +/- MPA (1 μM) (DM) for a maximum of 72 h. Black bars denote ESC from non-pregnant endometrium and chequered bars denote DSC from 1st trimester decidua. Graph A depicts cells treated for 2h. Graph B depicts cells treated for 12 h. No significant differences are seen between treatments. Graph C depicts cells treated for 24 h. Graph D depicts cells treated for 48 h and Graph E depicts cells treated for 72 h. dPRL mRNA is significantly potentiated in DSC compared to ESC after both were treated with DM for 24 h, ($p<0.01$), 48 h, ($p<0.001$) and 72 h, ($p<0.01$). Note graphs are on different scale axes to demonstrate differences at each time point. Graph F depicts cells treated with DM for a maximum of 72 h demonstrating the dPRL mRNA increase over time between ESCs and DSCs. n = 7 decidual biopsies and 7 endometrial biopsies.

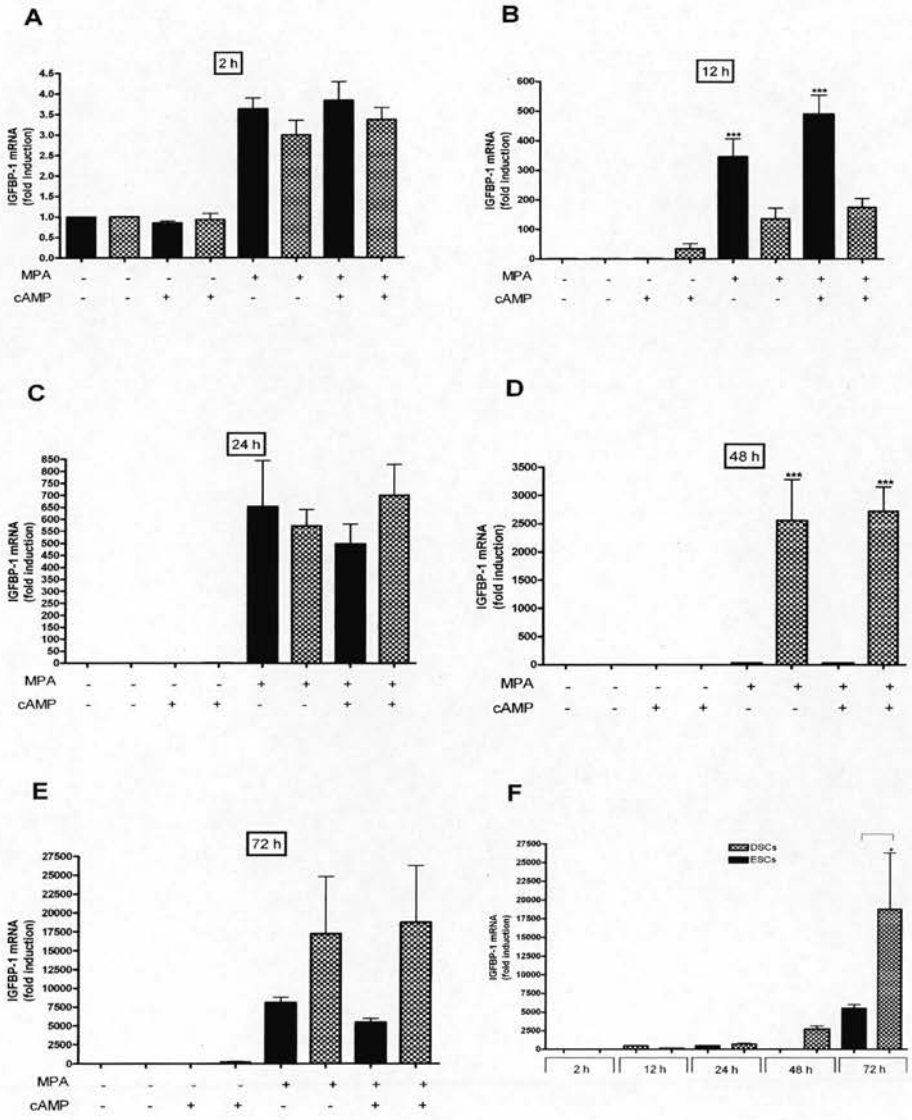


Figure 4.13: IGFBP-1 mRNA expression in DSC and ESC. DSC and ESC were cultured in the presence or absence of decidualising stimuli, cAMP (250 μM) +/- MPA (1 μM) for a maximum of 72 h. Black bars denote ESC from non-pregnant endometrium and chequered bars denote DSC from 1st trimester decidua. Graph A, no significant differences were seen between treatments after 2 h incubation. Graph B, IGFBP-1 mRNA was significantly potentiated in ESC compared to DSC after both were treated with MPA alone ($p<0.001$) or MPA + cAMP (DM) for 12 h, ($p<0.001$). Graph C, no significant differences were seen between treatments. Graph D, IGFBP-1 mRNA was significantly potentiated in DSC compared to ESC after both were treated with MPA alone ($p<0.001$) or DM for 48 h, ($p<0.001$). Graph E, no significant differences were observed between treatments. Note graphs are on different scale axes to demonstrate differences between ESCs and DSCs at each time point. Graph F depicts cells treated with DM for a maximum of 72 h demonstrating the IGFBP-1 mRNA increase over time between ESCs and DSCs. n = 7 decidual biopsies and 7 endometrial biopsies.

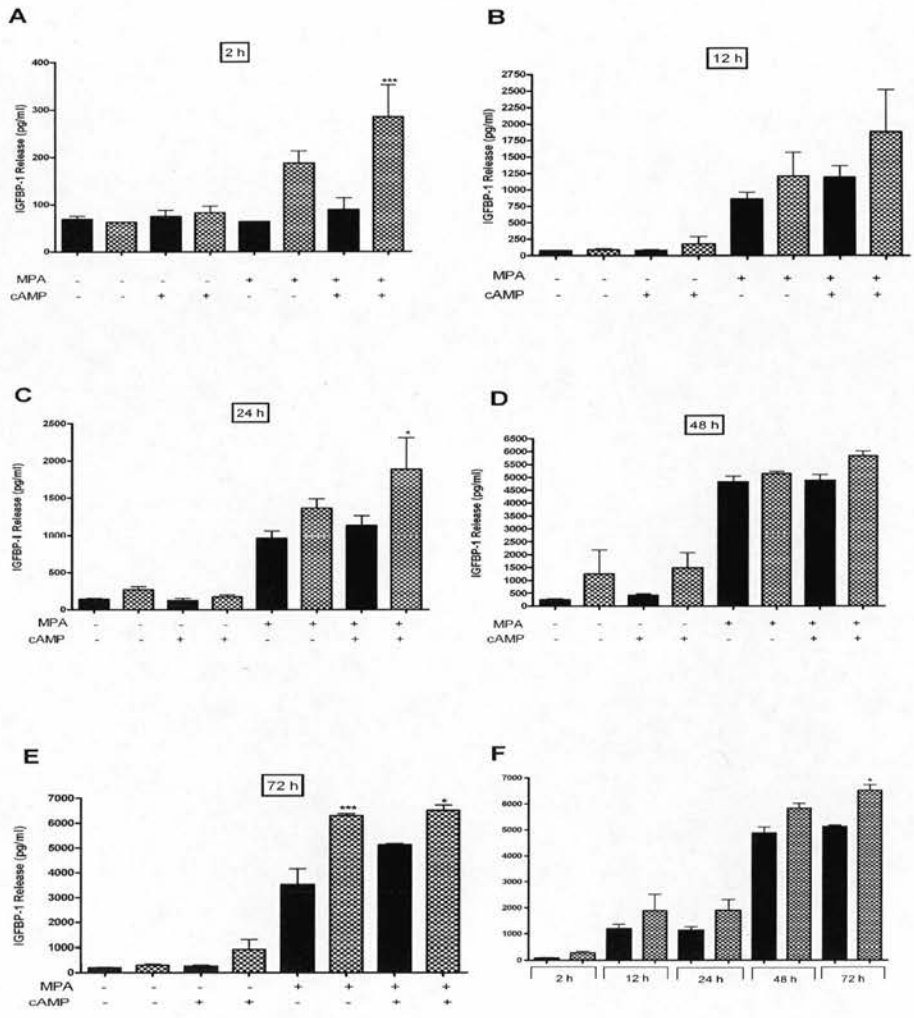


Figure 4.14: IGFBP-1 protein release from DSC and ESC. DSC and ESC were cultured in the presence or absence of decidualising stimuli, cAMP (250 μM) +/- MPA (1 μM) (DM) for a maximum of 72 h. Black bars denote ESC from non-pregnant endometrium and chequered bars denote DSC from 1st trimester decidua. Graph A, IGFBP-1 protein release is significantly potentiated in DSC compared to ESC after both were treated with DM for 2 h, ($p<0.001$). Graph B depicts cells treated for 12 h. No significant differences are seen between treatments. Graph C, IGFBP-1 protein release is significantly potentiated in DSC compared to ESC after both were treated with DM for 24 h, ($p<0.05$). Graph D depicts cells treated for 48 h. No significant differences were observed between treatments. Graph E, IGFBP-1 protein release is significantly potentiated in DSC compared to ESC after both were treated with MPA alone ($p<0.001$, n = 7 decidual biopsies) or DM for 72 h, ($p<0.05$). Note graphs are on different scale axes to demonstrate differences between ESCs and DSCs at each time point. Graph F depicts cells treated with DM for a maximum of 72 h demonstrating the IGFBP-1 mRNA increase over time between ESCs and DSCs. n = 7 decidual biopsies and 7 endometrial biopsies.

4.3.4.4 TGFβ1 suppresses the expression and release of decidualisation product genes

To address the question as to the effect of TGFβ1 on decidualisation genes produced by DSC, re-decidualised *in vitro*, cells obtained from < 10 wks gestation were cultured, decidualised *in vitro* with 8-Br-cAMP (0.5 mM) and MPA (1 μM) (decidualising medium) for 6 d and then further cultured with decidualising medium to maintain the decidualised phenotype with or without the addition of TGFβ1 for up to 72 h. Results are presented in Figures 4.15 to 4.19; 10 ng/ml TGFβ1 downregulated expression of dPRL at mRNA expression level in a time dependent manner (Figures 4.15), with significant down-regulation seen at 72 h (p,0.001, n = 7 decidual samples), as compared to unstimulated, time-matched controls. No significant effect was observed for dPRL protein release, but displayed a trend towards reduction, as measured by time-resolved fluoroimmunoassay (Figure 4.16). TGFβ1-induced inhibition of IGFBP-1 (Figure 4.17) ($p<0.05$, n = 7 decidual samples) and TF (Figure 4.19) ($p<0.05$, n = 7 decidual samples) mirrored that of dPRL, in that a significant reduction was only observed after 72 h of treatment. No significant effect was observed on IGFBP-1 mature protein secretion (Figure 4.18).

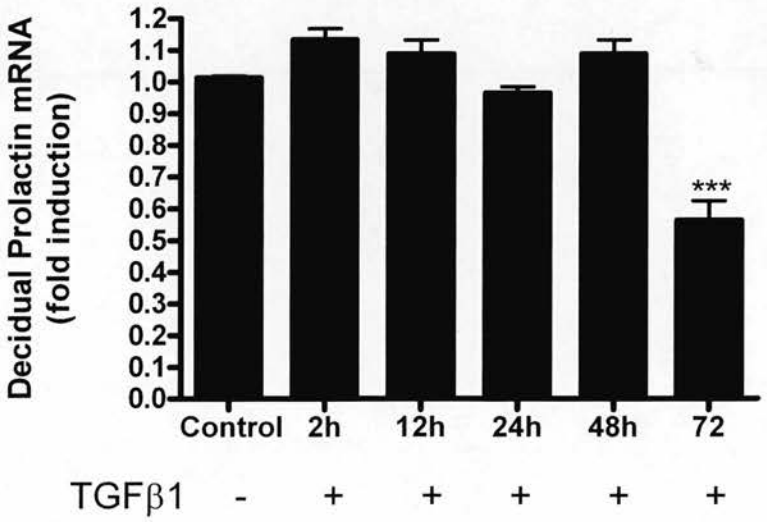


Figure 4.15: TGFβ1 inhibits expression of dPRL mRNA in DSC, as measured by Q-RT-PCR. DSCs were decidualised *in vitro* and treated in the presence or absence of TGFβ1 for a maximum of 72 h. TGFβ1 reduces expression of dPRL mRNA after 72 h treatment. ($p<0.001$, n = 7 decidual samples).

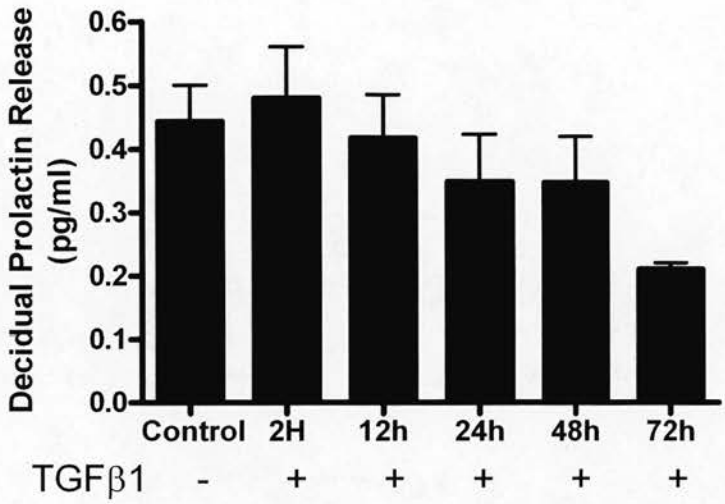


Figure 4.16: TGFβ1 is without effect on prolactin protein release, measured by time-resolved fluoroimmunoassay. Cells were decidualised *in vitro* and treated in the presence or absence of TGFβ1 for a maximum of 72 h. TGFβ1 was without significant effect on protein release of prolactin, although did display a trend toward inhibition (n = 7 decidual samples).

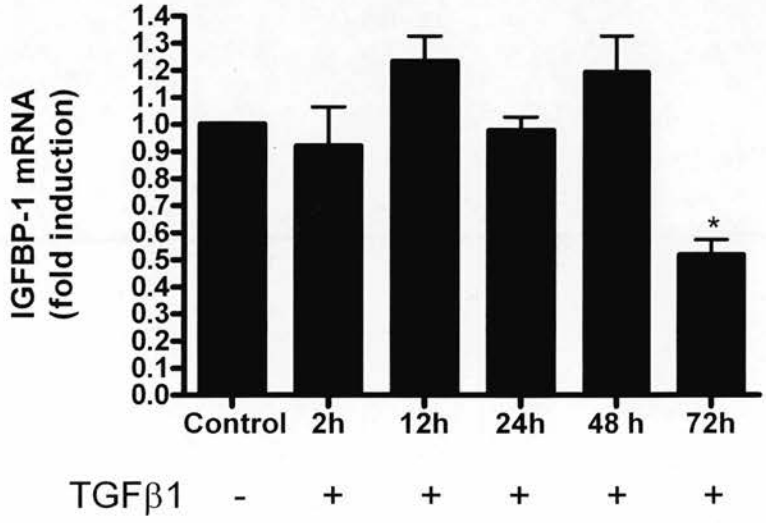


Figure 4.17: TGFβ1 inhibits expression of IGFBP-1 mRNA in DSC, as measured by Q-RT-PCR. DSCs were decidualised *in vitro* and treated in the presence or absence of TGFβ1 for a maximum of 72 h. TGFβ1 reduces expression of IGFBP-1 mRNA after 72 h treatment. ($p<0.05$, n = 7 decidual samples).

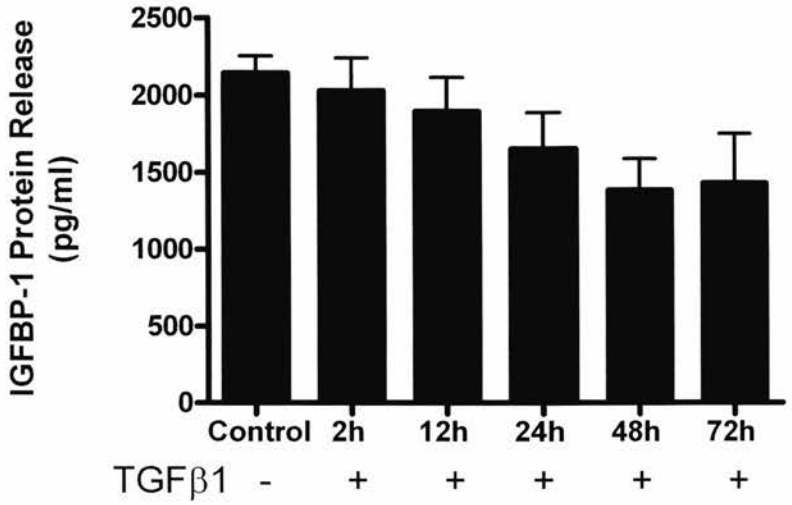


Figure 4.18: TGFβ1 is without effect on IGFBP-1 protein release, measured by ELISA. Cells were decidualised *in vitro* and treated in the presence or absence of TGFβ1 for a maximum of 72 h. TGFβ1 was without significant effect on protein release of IGFBP-1 (n = 7 decidual samples).

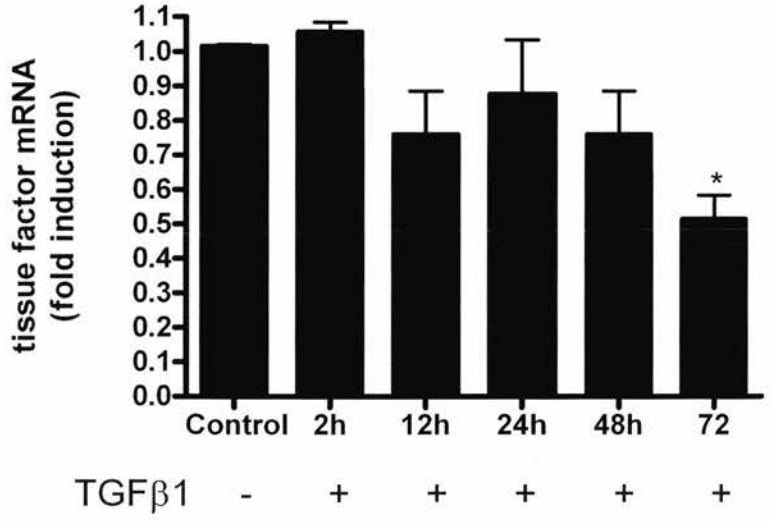


Figure 4.19: TGFβ1 inhibits expression of TF mRNA in DSC, as measured by Q-RT-PCR. DSCs were decidualised *in vitro* and treated in the presence or absence of TGFβ1 for a maximum of 72 h. TGFβ1 reduces expression of TF mRNA after 72 h treatment. ($p<0.05$, n = 7 decidual samples).

4.3.4.5 Effect of abrogating the SMAD signalling pathway on TGFβ1- induced inhibition of dPRL in ESC

SMAD 4 siRNA was used to investigate if selectively silencing the SMAD signalling pathway would abrogate the TGFβ1-incuded responses. ESCs were transiently transfected with either a non-targetting siRNA or SMAD 4 siRNA or were subjected to a mock transfection. 4 hours after transfection the cells were then subsequently cultured +/- 8-Br-cAMP (0.5 mM) and MPA (1 μM) +/- TGFβ1 (10 ng/ml) for 72 h. Successful silencing was assessed by detecting the SMAD 4 mRNA and protein levels by non-quantitative PCR and Western blotting respectively, results shown in section 3.3.9.

To elucidate if silencing SMAD 4 would abrogate the TGFβ1-induced effect on dPRL mRNA levels were measured. Results are presented in Figure 4.20; as observed previously, treatment with cAMP and MPA evoked an increase in dPRL mRNA expression. Transfection with SMAD 4 siRNA significantly suppressed dPRL mRNA as compared with the decidualised cells (Figure 4.20, $p<0.001$). Treatment with TGFβ1 with either the non-targeting siRNA or SMAD 4-specific siRNA transfected cells demonstrated a reduction in the level of dPRL mRNA in comparison with decidualised cells (Figure 4.20, $p<0.001$). This was suppressed further in the cells transfected with SMAD 4 siRNA (Figure 4.20). However, as transfection with the non-targeting siRNA significantly inhibited dPRL mRNA expression (Figure 4.20, $p<0.001$), no comparison can be drawn on the relevance of the SMAD signalling pathway in TGFβ1-induced inhibition of dPRL mRNA expression and protein release. The transient transfection and treatment of the cells was performed by Marius Jones in Jan Brosen's lab.

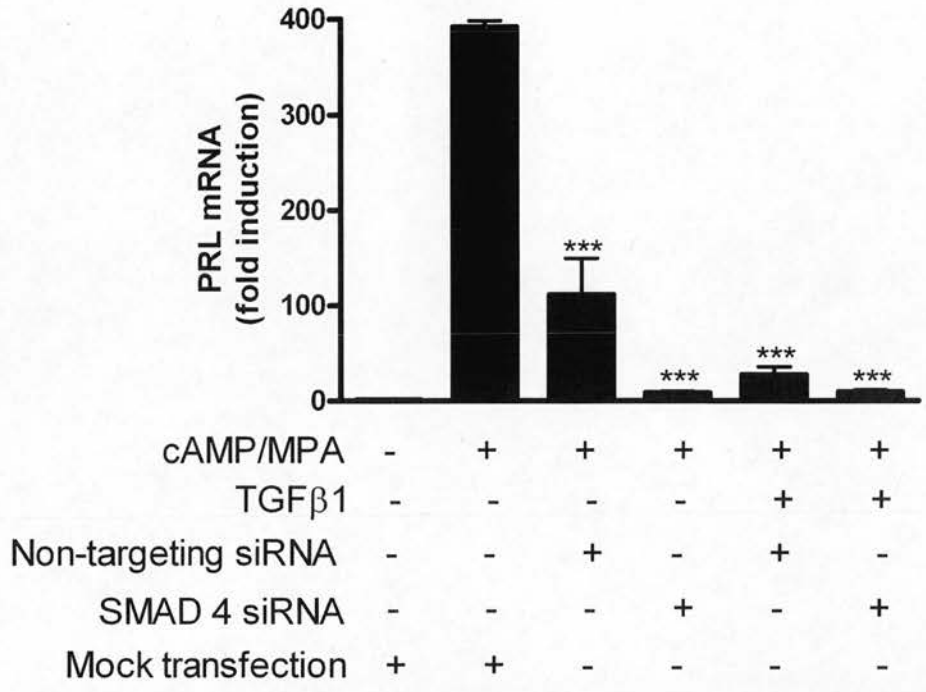


Figure 4.20: Cultured ESCs were transiently transfected with either control siRNA or SMAD 4 siRNA and subsequently cultured for 72 h, +/- 8-Br-cAMP (0.5 mM) and MPA (1 μM) +/- TGFβ1 (10 ng/ml). Treatment with decidualising stimuli evoked an increase in PRL mRNA. A significant reduction was observed with cells transfected with the non-targeting siRNA and SMAD 4 siRNA, both treated with and without TGFβ1 ($p<0.001$). n = 1 endometrial sample.

4.4 Discussion

We have demonstrated that TGFβ1 markedly inhibits the expression of mRNA's encoding the decidual protein markers, PRL (Christian, Marangos et al. 2001), IGFBP-1, and tissue factor (Figures 4.1, 4.3 and 4.5), and that this inhibitory effect at the level of mRNA was reflected by a reduction in mature protein secretion of both PRL and IGFBP-1 (Figures 4.2 and 4.4) which was in agreement with previous published data (Mazella, Tang et al. 2004). It would appear that the normal protein versus mRNA kinetics are not as expected which would suggest that TGFβ1 may also be repressing translation/export of protein processing. A number of studies have reported a marked inhibitory effect of TGFβ1 on basal and stimulated PRL secretion, mRNA levels and *de novo* PRL synthesis in rat anterior pituitary cells (Herrlich,

Kuhn et al. 1996; Telgmann, Maronde et al. 1997; Coya, Alvarez et al. 1999). These authors report a time-dependent dual effect: a short-term inhibition (after 4 h) of basal prolactin-secretion and a long-term inhibition (after 4 d) of basal and stimulated prolactin expression. They also demonstrate that the short-term effect of TGFβ1 was independent of cAMP reduction, and via pertussis toxin abolition of inhibitory actions of TGFβ1, implicate the involvement of G-proteins as signal transducers of TGFβ1 prolactin inhibition (Coya, Alvarez et al. 1999). It was suggested that the long-term effect of TGFβ1 as regards PRL inhibition might be attributed to decreased cAMP levels. Indeed, initiation of the decidual process in endometrial stromal cells both *in vivo* and *in vitro* requires elevated intracellular cAMP levels (Tanaka, Miyazaki et al. 1993; Herrlich, Kuhn et al. 1996; Telgmann, Maronde et al. 1997). Telgmann, *et al* (Telgmann and Gellersen 1998), observed that the major activation of the decidual PRL promoter depends upon the region located between –332 bp and –270 bp relative to the transcription site, demonstrating that deletion of this region significantly reduced cAMP induction. Subsequently, this essential region was shown to contain a progesterone response element (PRE) half site (Christian, Pohnke et al. 2002). The data presented herein confirms that TGFβ1 abrogates the transactivation potential of the dPRL promoter-reporter, by more than 50%, acting at the minimal PKA-sensitive decidual PRL promoter construct (dPRL-332/Luc), in response to cAMP- treated endometrial stromal cells (Figure 4.6). This suggests that TGFβ1 acts upon the region critical for successful transcription.

In contrast to the findings of the present studies, Celikkanat *et al* (Celikkanat, Atac et al. 2000), have reported that there is no direct effect of TGFβ1 on the differentiation of human ESC and on the production of dPRL. It would be unlikely that they would have found any results to the contrary as the cells were not decidualised *in vitro* and as such would only produce basal levels of prolactin. They do agree with the suggestion, that TGFβ1 may be involved in a more complex role within regulation of decidualisation. Also in contrast to the present data, Kim *et al* (Kim, Park et al. 2005), propose that TGFβ1 actually potentiates the decidualisation process in ESC and report that progesterone is not required to induce decidualisation in ESC (Kim,

Park et al. 2005). It must be noted that this is the only study to suggest that decidualisation is a progesterone-independent event.

Previous studies have identified TGFβ1 as an inhibitor of the relaxin receptor (LGR7) and of IGFBP-1 production; both recognised markers of decidualisation. The findings herein are in agreement with those of Mazella et al (Mazella, Tang et al. 2004) who also reported that TGFβ1 inhibited both IGFBP-1 mRNA and protein production in a time-dependent manner and Vićovac et al and Jikihara et al (Jikihara and Handwerger 1994; Vicovac, Starkey et al. 1994), who have both demonstrated a TGFβ1 dose-dependent inhibition of PRL by decidual stromal cells derived from first trimester pregnancy and decidual cells from term pregnancy, respectively. The cells in our study were obtained from non-pregnant endometrium and decidualised *in vitro* and are more indicative of the cells which are seen to decidualise in the absence of a blastocyst *in vivo*.

Tierney *et al* (Tierney, Tulac et al. 2003) reported that expression of TGFβ1 is induced in response to cAMP within 12-24 h and suggested that TGFβ1 is primarily involved in cellular differentiation and the adoption of the morphologic and secretory phenotype of the decidual stromal cell. However, due to the microarray-based nature of Tierney's findings, they only report on mRNA levels and not protein, therefore, it is unclear as to whether the cAMP-induced TGFβ1 is latent or active. It is possible that activation of TGFβ1 may occur with cell-to-cell contact and may act locally to inhibit the progression of decidualisation. Aberrant regulation of TGFβ1 may contribute to menstrual disorders, such as heavy menstrual bleeding and dysmenhorrea, by preventing haemostasis and causing infertility by preventing decidualisation of the endometrium.

Previous studies have sought to identify an *in vitro* model better placed to study the effects and induction or inhibition of decidualisation (Schatz and Lockwood 1993). Cultured 1st trimester decidual cells have been utilised and reported to retain key morphological features indicative of their decidualised *in vivo* state, for example,

they are reported to form multicellular clusters surrounded by patches of pericellular basement membrane type ECM (Schatz, Papp et al. 1994), however the cells were cultured in the presence of decidualising stimuli so it is impossible to ascertain if the cells are capable of de-differentiating (Schatz, Papp et al. 1994). In our study, experiments to determine if stromal cells from 1st trimester decidua de-differentiate indicate that although the cells are seen to de-differentiate morphologically (Figure 4.10) there is still a notable production of the classic decidualisation marker, IGFBP-1, even after 14 d of culture in the absence of decidualising stimuli (Figure 4.9, Panel A). This could be in part due to individual variability and differences in gestation between samples. For the most part, DSCs from gestations > 10 weeks take longer to release reduced concentrations of IGFBP-1 and may need a longer time in culture to de-differentiate (Figure 4.9, Panel B). Furthermore, DSCs re-decidualise faster than ESC when treated in parallel (Figures 4.12 – 4.14), implying the presence of a potential "decidualised phenotype memory" when cells are decidualised *in vitro*. Indeed, DSCs display an increased sensitivity to MPA and can re-decidualise in response to *in vitro* MPA treatment alone after a shorter treatment period than ESC (Figure 4.13 and 4.14).

In addition, the present studies have demonstrated that TGFβ1 markedly inhibits the expression of dPRL, IGFBP-1, and tissue factor mRNAs in DSCs (Figures 4.15, 4.17 and 4.19). However, this downregulation was delayed by at least 24 h when compared to the response seen in ESC. Furthermore, this inhibitory effect at the level of mRNA was not reflected by a reduction in mature protein secretion of both PRL and IGFBP-1 in DSC (Figures 4.16 – 4.18), implying that decidualisation *in vivo*, confers some resistance to the actions of TGFβ1. Perhaps the presence of blastocyst and increasing concentrations of hCG in the 1st trimester of pregnancy evokes an increase in cellular protection against potential pathogenic cytokines and growth factors, or perhaps the role of TGFβ1 in pregnant endometrium differs from that in non-pregnant, pre-menstrual endometrium. This would seem to be in agreement with data reporting high expression of TGFβ1 in 1st trimester decidua without any detrimental effect to pregnancy (Lysiak, Hunt et al. 1995; Jokhi, King et al. 1997;

Bennett, Lagoo-Deenadayalan et al. 1999; Simpson, Robson et al. 2002). Indeed, maintaining homeostasis in decidua is of paramount importance to ensure the successful continuation of pregnancy. Aberrant increases in active TGFβ1 may be detrimental for the developing foetus.

In summary, the studies presented in this chapter have demonstrated that TGFβ1 is capable of suppressing the decidualisation marker proteins. The results obtained suggest that ESCs isolated from non-pregnant endometrium are a better model than DSCs isolated from 1st trimester decidua for evaluating the events which occur in the premenstrual endometrium, whereas the DSCs are a better model for evaluating the events which occur in the presence of a blastocyst. The findings also suggest that local TGFβ1 signalling may coordinate de-differentiation of endometrial stromal compartment and tissue remodelling associated with menstruation, but may have a different role in pregnant endometrium.

Chapter 5

5 Uterine natural killer cell interactions with human chorionic gonadotrophin

5.1 Introduction

5.1.1 Uterine natural killer cells

$CD56^{bright}$ lymphocytes (uterine natural killer cells; uNKs) increase in number in the human uterus during every menstrual cycle (King 2000). The increase in uNKs follows the surge in pituitary-derived luteinising hormone (LH), and is coincidental with the increase in progesterone concentration during the secretory phase of the cycle. While the uterus is host to some $CD56^{bright}$ cells prior to ovulation, the rapid increase is thought to be due to proliferation of the resident population, accompanied by recruitment of $CD56^{bright}$ lymphocytes from the circulation. The rapid increase in $CD56^{bright}$ cells is concurrent with the onset of decidualisation. Uterine $CD56^{bright}$ cells are proposed to proliferate and differentiate to become the predominant lymphocytes of the post-ovulatory uterus (Trundley and Moffett 2004). These distinct, tissue-specific natural killer (NK) cells either decrease in number prior to menses or increase in number during early pregnancy, and then decline toward the end of the first trimester. The exact function of uNK cells remains unknown, however, reduced NK cell populations are reported in pregnancies complicated by foetal growth restriction (FGR) with or without the accompanying condition, pre-eclampsia (Eide, Rolfseng et al. 2006).

Ultrastructurally, a typical uterine $CD56^{bright}$ cell is a large lymphocyte with a reniform, eccentric nucleus and short, cytoplasmic projections. They also possess varying numbers of membrane-bound cytoplasmic granules that contain cytolytic molecules such as granzyme and perforin (Trundley and Moffett 2004). $CD56^{Bright}$

populations respond differentially to chemotactic signalling; $CD56^{dim}$ cells migrate in response to CXCL8 (IL-8) and strongly express the receptor CXCR1. $CD56^{bright}$ cells express the chemokine receptors CCR5, which binds CCL3 (MIPα1), CCL4 (MIPβ1) and CCL5 (RANTES), and CCR7, which binds CCL19 (MIPβ3) and CCL21 (Campbell and Colonna 2001). uNK cells have a unique expression pattern, strongly expressing, CCR1, 2, and 5, CXCR3 and 4, and CX3CR1 (Red-Horse, Drake et al. 2001). The expression of both phenotypic and chemokine receptors for each $CD56^{+}$ population is summarised in Table 5.1.

Receptors	**Blood $CD56^{Dim}$**	**Blood $CD56^{Bright}$**	**uNK**
CD56	+	++	+++
CD16	+	–	–
CD3	–	–	–
L-Selectin	+	++	–
Alpha 4 integrin	+	++	++
LFA-1	+	+	+
CCR1	–	–	++
CCR2	–	–	++
CCR5	–	++	++
CCR7	–	++	+/–
CXCR1	+	–	–
CXCR2	+	–	–
CXCR3	+	++	++
CXCR4	++	++	++
CX3CR1	+	++	++

Table 5.1: The expression pattern of both phenotypic and chemokine receptors for each $CD56^{+}$ population. Adapted from Van Den Heuvel *et al* (van den Heuvel, Peralta et al. 2005).

(+) Expressed; (++) strongly expressed; (+++) very strongly expressed; (–) not expressed.

5.1.2 Macrophage Mannose Receptor

Macrophage mannose receptors (MMRs) are multivalent transmembrane receptors capable of interacting with multiple ligands. The structure of the MMR is further discussed in section 1.4.3. MMRs are expressed by most tissue macrophages, but are not macrophage-restricted (Shepherd, Tarnowski et al. 1991; Linehan, Martinez-Pomares et al. 1999; Wilt, Greaton et al. 1999; Engering, Geijtenbeek et al. 2002; Taylor, Gordon et al. 2005). A mannose-acetate-specific receptor was previously identified on PBNK cells, and shown to be responsible for NK activity (Hauer, Voetsch et al. 1994), however no studies have yet been undertaken to analyse the uNK cell population for MMR. MMRs are able to bind foreign and endogenous molecules (sulphated glycoproteins or glycoproteins containing mannose, fucose and N glucosamine residues) in a Ca^{2+} dependent manner and thereafter endocytose them by clathrin-mediated endocytosis (East, Rushton et al. 2002). MMRs are recycled between the plasma membrane and the endosomal apparatus to mediate the uptake of extracellular ligands (East and Isacke 2002). The MMR may also mediate phagocytosis of a wide variety of microbes including fungi, protozoa, yeast and bacteria, all of which display a high mannose cell surface expression (Taylor, Bezouska et al. 1992). MMRs have also been demonstrated to bind sulphated oligosaccharides, such as those present on the pituitary hormones LH and TSH (Fiete, Beranek et al. 1998; Simpson, Hitchen et al. 1999). hCG and LH are both oligosaccharide glycoproteins (Acevedo 2002), with identical α subunits, which act via their common receptor (Rajaniemi, Ronnberg et al. 1981), and are recognised to regulate non-gonadal and immune cell function (Rao 2001). For instance, LH has previously been shown to suppress the lytic activity of PBNK cells (Sulke, Jones et al. 1985) and to increase the adhesion of PBNK cells to endothelium (van den Heuvel, Peralta et al. 2005), however the LH/hCG receptor was never localised to these cells (Sulke, Jones et al. 1985; van den Heuvel, Peralta et al. 2005). Recent studies have localised the LH/hCG receptor to macrophages in late secretory phase endometrium and term placenta (Zhang, Rao Ch et al. 2003). It is unknown whether the MMR can bind and internalise hCG or LH, however, a previous study

demonstrated that high doses of hCG promoted IL-8 production by peripheral blood mononuclear cells (PBMC)s in the absence of detectable LH/hCG receptors (Kosaka, Fujiwara et al. 2002). The authors further demonstrated that this production could be abrogated by the addition of D-mannose and postulated that hCG effects on PBMCs may be mediated via a C-type lectin (Kosaka, Fujiwara et al. 2002).

5.1.3 Hypothesis

In summary, previous studies have demonstrated a functional role of LH in mediating NK lytic activity (Sulke, Jones et al. 1985) and adhesion properties (van den Heuvel, Peralta et al. 2005). However, no NK locus has been identified for the LH/hCG receptors in PBNK cells (Sulke, Jones et al. 1985; van den Heuvel, Peralta et al. 2005). With the expansion of $CD56^{Bright}$ uNK cells in the uterus following the surge in LH (King 2000), and the maintenance of uNK cell numbers in 1st trimester endometrium in accordance with hCG levels in maternal plasma (**Jaffe, Lee et al. 1969; King 2000**) it is unknown whether uNK cells express the LH/hCG receptor, hence providing a possible mechanism for the recruitment and/or proliferation of this cell population *in utero*. MMRs have been demonstrated to bind sulphated oligosaccharide glycoproteins (Fiete, Beranek et al. 1998; Simpson, Hitchen et al. 1999) and both hCG and LH are both oligosaccharide glycoproteins (Acevedo 2002) therefore it could be hypothesised that MMR may bind hCG and LH, so, does the uNK cell possess MMRs?

5.1.4 AIMS

The studies described in this chapter were designed to investigate uNK cell interactions with the maternal recognition of pregnancy hormone, hCG. All studies utilised primary uNK cells isolated from 1st trimester decidua and non-pregnant late-secretory endometrium as described in section 2.1 and 4.2.1.2. Experiments in this chapter were designed to address the following questions:

1: Do uNK cells express the LH/hCG receptor?

2: Does treatment with hCG induce proliferation of uNK cells derived from 1st trimester decidua?

3: Does treatment with hCG effect production of uNK-associated cytokines, IL-10, TNFα and IFNγ?

4: Do uNK cell possess MMRs?

5: Does hCG colocalise with MMR?

5.2 Methods

5.2.1 Human uterine tissue collection

5.2.1.1 Tissue collection and processing of human decidual tissue

Decidual samples (n = 46) were collected as detailed in section 2.1.1 (Table 2.1b). Decidual material utilised in this chapter are detailed in Table 5.1. All decidual samples were processed to isolate the $CD56^{bright}$ uterine natural killer cells (uNKs) as described in section 4.2.1.2. Cells were resuspended in 80 ml of MACS™ buffer (500 ml PBS pH 7.2, 0.5 % BSA and 2 mM EDTA) (Miltenyi Biotec), to which 20 µl of CD56 microbeads (Miltenyi Biotec) were added. Thereafter, the cells were washed in 10 ml MACS™ buffer (Miltenyi Biotec) and centrifuged at 400 g for 5 min. The supernatant was discarded. 500 µl of MACS™ buffer (Miltenyi Biotec) was added to 108 cells. This mixture was subsequently passed through a 30 µm filter to ensure no cell clumps. The MACS™ LD column (Miltenyi Biotec) was placed in the magnetic field of the MACS™ separator (Miltenyi Biotec) and primed by rinsing with 2 ml of MACS™ buffer. The cell suspension was then passed through the columns. The unlabelled cells passed through the column and were thereafter treated as described in section 4.2.1.2. The labelled cell fraction was eluted from the column and treated as described in section 5.2.2.

5.2.1.2 Late secretory endometrium from non pregnant women

An endometrial sample was collected as detailed in section 2.1.1 (Table 2.1a), and was processed to isolate the $CD56^{bright}$ uterine natural killer cells (uNKs) as described in section 4.2.1.2. The sample included superficial and basal endometrium. The patient, aged 45, described regular menstrual cycles (25-35 days) and had not received any form of exogenous hormonal treatment in the 3 months preceding biopsy. The biopsy was dated as day 27 of the menstrual cycle according to the patient's reported last menstrual period (LMP) and was included for analysis as the histological dating according to published criteria (Noyes, Hertig et al. 1950) and circulating sex steroid concentrations (E_2 and P at time of endometrial biopsy) were consistent with the LMP. Serum was separated (by centrifugation at 800 g for 7 min) from venous blood samples collected at the time of biopsy and E_2, 323 pg/ml and P, 27 ng/ml, concentrations were measured by RAI. The tissue sample was collected under Ethics submission number 17 (Appendix).

Sample	D.O.C	AGE	PARITY LB	PARITY MT	Gestation (Wks + day)	Ethical approval ref
1	14/04/04	28	1	0	8+4	73
2	01/11/04	23	0	0	9+3	73
3	10/11/04	20	1	0	7+5	73
4	15/11/04	36	3	0	8+3	73
5	15/11/04	31	0	0	7+6	73
6	24/11/04	36	1	0	7+6	73
7	24/11/04	23	0	1	8+4	73
8	29/11/04	30	2	2	8+3	73
9	01/12/04	23	2	0	7+6	73
10	25/01/05	24	2	1	11	73
11	13/09/05	26	2	0	8	05/S1104/12
12	13/09/05	21	0	0	8	05/S1104/12
13	14/09/05	43	2	1	7+4	05/S1104/12
14	27/09/05	26	0	0	8+6	05/S1104/12
15	27/09/05	21	0	0	9+5	05/S1104/12
16	28/09/05	43	0	1	8	05/S1104/12
17	28/09/05	26	1	0	9+2	05/S1104/12
18	29/09/05	23	0	0	8+5	05/S1104/12
19	29/09/05	21	0	0	9+1	05/S1104/12
20	04/10/05	30	0	0	9+3	05/S1104/12
21	04/10/05	18	1	1	8+3	05/S1104/12
22	12/10/05	26	2	0	9+3	05/S1104/12
23	12/10/05	20	1	4	9+6	05/S1104/12
24	26/10/05	24	0	0	11+1	05/S1104/12
25	28/11/05	30	3	1	10+2	05/S1104/12
26	29/11/05	24	0	0	11	05/S1104/12
27	02/12/05	27	1	1	10+3	05/S1104/12
28	02/12/05	28	3	0	8+2	05/S1104/12
29	05/12/05	23	1	1	10+4	05/S1104/12
30	13/12/05	24	0	0	9+5	05/S1104/12

31	16/02/06	36	0	0	10+3	05/S1104/12
32	21/02/06	21	0	0	8+1	05/S1104/12
33	22/02/06	19	0	1	10+2	05/S1104/12
34	02/03/06	25	0	0	10	05/S1104/12
35	02/03/06	43	2	0	9+2	05/S1104/12
36	02/03/06	18	1	0	9+6	05/S1104/12
37	09/03/06	19	0	0	11	05/S1104/12
38	15/03/06	25	0	0	10+4	05/S1104/12
39	15/03/06	25	0	0	< 8	05/S1104/12
40	20/03/06	20	0	0	10+1	05/S1104/12
41	20/03/06	42	1	0	8+6	05/S1104/12
42	22/03/06	20	0	0	9+2	05/S1104/12
43	22/03/06	34	0	0	8	05/S1104/12
44	22/03/06	30	1	1	9+6	05/S1104/12
45	22/03/06	18	0	0	8	05/S1104/12
46	22/03/06	26	2	0	9+5	05/S1104/12

Table 5.2: Details of 1st trimester decidual biopsies used in the studies presented in Chapter 5. D.O.C. = Date of Collection. Parity LB = number of live births. Parity MT = number of miscarriage/terminations.

5.2.2 *In vitro* primary cell culture experiments

uNK cells were treated with hCG (10 ng/ml – 10000 ng/ml) in RPMI 1640 medium (Sigma) supplemented with 10 % FCS (Mycoplex), penicillin (50 μg/ml; Sigma), streptomycin (50 μg/ml; Sigma) and gentamycin (5 μg/ml; Sigma) for 2 h at 37°C in 5 % (v/v) CO_2. The cells were seeded in 6-well plates at a concentration of 2.5 x 10^6 cells/ml. Each treatment was duplicated and repeated 9 times with separate decidual biopsies.

5.2.3 RNA extraction

Total RNA was extracted from the uNK cells as described in section 2.3.1 and cDNA prepared from the experiments detailed in section 5.2.2 as described in section 2.3.2.

5.2.4 Semi-Quantitative PCR

Primers were designed to amplify the LH/hCG receptor or MMR cDNAs. PCR was carried out using BioMix Red (Bioline). Conditions for each sample were as follows:

2 x Bioline Biomix Red	10μl
LH/hCG receptor/ MMR sense primer (0.5 μM)	2 μl
LH/hCG receptor/ MMR antisense primer (0.5 μM)	2 μl
RNAse-free H_2O	2 μl
cDNA	4 μl

30 cycles of amplification were performed with an initial denaturing temperature of 95°C for 5 min for 1 cycle followed by 30 cycles of: denaturation at 95°C for 30 sec, annealing for 30 sec and extension at 72°C for 1 min 30 sec. Final extension at 72°C was carried out for 10 min. The PCR product was run on a 2% agarose gel (section 2.4.1), visualised, and recorded as described in section 2.4.1. Primer pairs designed to amplify LH/hCG receptor, MMR and GAPDH cDNAs are given in Table 5.3. Amplified products were visualised on agarose gels as described in section 2.4.1.

Amplicon	Accession Number	Forward Primer	Reverse Primer	Annealing Temp °C	Product size (bp)
LH/hCG receptor	NM_000233	ATGAAGC AGCGGTT CTCG	TTGACAGG GAGGTAG GCAAG	62	203
MMR	X55635	CTACCCC TGCTCCT GGTTTTT	TGAAACAC TCATAATC TGAGATTC	60	203
GAPDH	NM_002046	CTGCACC ACCAACT GCTTAGC	ATGCCAGT GAGCTTCC GTTC	58	205

Table 5.3: Primer sequences and product sizes

5.2.5 Q-RT-PCR

The relative levels of expression of IL-10, IFNγ and TNFα mRNAs in uNK cells, (control and treated cells; section 5.2.2), were determined using quantitative RT-PCR (TaqMan) using random primed cDNA as detailed in section 2.4.2. Results were analysed as in section 2.4.2. Primer pairs and probes specific to each gene are given in Table 5.4. Primer pairs and probes were designed by Dr Elena Faccenda, a member of the laboratory group.

Amplicon	Accession Number	Forward Primer	Reverse Primer	Probe
IL-10	M57627	CTACGGC GCTGTCAT CGAT	TGGAGCTTA TTAAAGGCA TTCTTCA	CTTCCCTGTG AAAACAAGA GCAAGGCC
IFNγ	X13274	CCAACGCA AAGCAATA CATGA	TTTTCGCTT CCCTGTTTT AGCT	CTCATCCAAG TGATGGCTGA ACTGTCGC
MMR	X55635	TGCGACAG TAAACGAG GCTACA	TCGTTGCTGG AGGATTAGT CAA	ATGCCAGACA CGATCCGACC CTTC
TNFα	X02910	GGAGAAGG GTGACCGA CTCA	TGCCCAGACT CGGCAAAG	CGCTGAGATC AATCGGCCCG ACTA

Table 5.4: Primer and probe sequences

5.2.6 ELISA

5.2.6.1 IL-10 and TNFα

Media collected from uNK cell cultures was assayed for IL-10 and TNFα using a two-site sandwich ELISA as described in section 2.6.7.1. Plates were coated with the human IL-10 capture antibody (Pharmingen, Beckton Dickinson Company, Oxford, UK) (2 μg/ml, 100 μl per well) or the human TNFα capture antibody (R&D Systems) (2 μg/ml, 100 μl per well) and incubated overnight at 4°C. The plates were washed prior to use. A standard curve for the IL-10 and TNFα assays, both consisting of

seven standards, 500 to 7.8 pg/ml and 2000 – 31.2 pg/ml respectively, was added in duplicate to each plate. The standards were prepared as described in section 3.2.9. The non-specific binding control (NSB), standards, and media samples to be assayed were added in duplicate, 100µl per well. The remainder of the assay was conducted as detailed in section 3.2.9.

5.2.6.2 IFNγ

Media collected from uNK cell cultures was assayed for IFNγ using a two-site sandwich ELISA as described in section 2.6.7.1, except that plates were pre-coated with antibody (Human IFNγ Immunoassay, R&D Systems). Microplates were coated with Assay Diluent RD1-51 (100 µl per well) (provided with kit). A standard curve consisting of eight standards, 1000 to 7.8 pg/ml, was added in duplicate to each plate. The standards were prepared as described in section 3.2.9. The NSB, standards, and media samples to be assayed were added in duplicate, 100 µl per well. The microplate was covered with the adhesive strip provided and incubated for 2 h at room temperature. Excess media was discarded and plates washed 4 times in wash buffer prior to addition of IFNγ Conjugate (200 µl per well) (provided with kit).The plate was incubated for 2 h on a plate shaker at room temperature, after which the plate was washed as aforementioned. Subsequently, 200 µl of substrate solution (provided with kit) was added to each well and incubated in a dark environment, at room temperature for 30 min. The reaction was stopped by addition of 50 µl/well Stop Solution (provided with kit). The colour was then measured on a plate reader at 450 nM. The details of the ELISA are summarised in Figure 2.3.

5.2.7 Immunocytofluorescence

uNK cells were prepared for immunostaining by Shandon Cytospin® (Thermo Electron Corporation, Cheshire, UK). Briefly, the slides and cardboard filters were placed into appropriate slots in the cytospin with the cardboard filters facing the centre of the cytospin, being sure that each filter and slide pair were flush with each other and that the hole in the filter was in position to permit the cells to smear the

slide. 100 µl of each sample was aliquoted into the appropriate wells of the cytospin and spun at 80 g for 3 min. After which, the cardboard filter was removed with care to ensure that the cell smear was not disturbed. The slides were allowed to dry overnight at room temperature, protected from dust. Cells were fixed in methanol for 10 min at room temperature prior to immunostaining.

5.2.7.1 CD56 immunoreactivity

CD56 immunocytofluorescence was performed using the protocol described in section 2.6.10. Briefly, 1st trimester uNK cells were prepared as described above. Endogenous peroxidase was blocked by incubating the slides with 3 % H_2O_2 in 60 % (v/v) methanol for 30 min at room temperature on a rocker and and non-specific binding sites of the primary antibody were in a 1:5 dilution of NGS in PBS containing 5 % BSA (NGS PBS/BSA). Endogenous avidin/biotin was blocked using a commercially available avidin-biotin blocking kit (Vector Laboratories Ltd, Peterborough, UK) then washed twice with PBS. Slides were incubated at 4°C overnight in a 1:250 dilution of mouse monoclonal anti-human CD56 (Zymed) NGS/ PBS/BSA. After washing once with PBS/PBS Tween and once with PBS/PBS for 5 min each, CD56 antibody binding was detected by applying a 1:500 dilution of peroxidase goat anti-mouse antibody (Dako UK Ltd) in NGS/PBS/BSA for 30 min followed by a tyramide Cy3 (Perkin-Elmer Life Sciences), diluted 1:50 in the buffer supplied for 10 min each at room temperature. After washing the slides as described above, cells were counterstained with To Pro™ (Molecular Probes) (1:2000 in PBS) for 2 min at room temperature. The slides were mounted under a glass coverslip using Permafluor™ mounting medium (Beckman Coulter). Immunofluorescence was visualised and recorded as described in section 2.6.9.10.

5.2.7.2 hCG and MMR immunoreactivity

hCG and MMR immunocytofluorescence was performed using the protocol described in section 2.6.10. Briefly, 1st trimester uNK cells were prepared as described above. Slides were incubated at 4°C overnight in a 1:20 dilution of rabbit

polyclonal anti-human hCG (Abcam) NGS/ PBS/BSA. After washing once with PBS Tween and once with PBS for 5 min each, hCG antibody binding was detected by applying a 1:500 dilution of biotinylated goat anti-rabbit antibody (Dako UK Ltd) in NGS/PBS/BSA for 30 min followed by streptavidin 546 (Molecular Probes) for 60 min each at room temperature. After washing the slides as described above, slides were incubated at 4°C overnight in a 1:10 dilution of mouse monoclonal anti-human MMR (Abcam) NGS/PBS/BSA. Anti-MMR antibody binding was detected by applying a 1:500 dilution of biotinylated goat anti-mouse antibody (Dako UK Ltd) in NGS/PBS/BSA for 30 min followed by streptavidin 488 (Molecular Probes) for 60 min each at room temperature. After washing the slides as described above, cells were counterstained with To Pro™ (Molecular Probes) (1:2000 in PBS) for 2 min at room temperature. The slides were mounted as described in section 5.2.7.1. Immunofluorescence was visualised and recorded as described in section 2.6.10.10.

5.2.7.3 Haematoxylin and Eosin staining

Haematoxylin and Eosin staining was performed as described in section 2.6.9.10 with the exception that slides were immersed in Eosin for 3 min after developing the nuclei in Scott's tap water. Thereafter, slides were dehydrated in increasing concentrations of alcohol and cleared in histoclear and xylene for 5 min each before mounting under a borosilicate glass coverslip (VWR) using Pertex™ (Cell Path, UK), a solvent based glue.

5.2.8 FACS analysis of uNK cell surface marker proteins

FACS analysis was using performed as described in section 2.7.1 using the antibodies detailed in Table 5.5.

For FACS analysis using both MMR and CD56 antibodies, 6 eppendorfs containing 5 x 10^5 cells each were re-suspended in 100 µl FACS buffer (PBS, containing 0.1 % N_aN_3 and 1 % FCS): the first tube remained untreated, 10 µl anti-mouse IgG whole molecule with FITC conjugated (Sigma) or PE conjugated was added to the second

and third tube respectively, 10 μl of the anti-CD56 antibody was added to the fourth tube, 20 μl of the anti-MMR antibody was added to the fifth tube and 10 μl of the anti-CD56 antibody and 20 μl of the anti-MMR antibody was added to the sixth tube. The remainder of the protocol was conducted as in section 2.7.1.

Antigen	Dilution	Species raised in	Source
CD56	1:10	Mouse	Serotec
MMR	1:5	Mouse	Serotec

Table 5.5: Antibodies used in FACS anaylsis.

5.2.9 Statistical Analysis

Prior to statistical analysis, all data was tested for and shown to demonstrate Gaussian distribution. Where appropriate, values were presented as means ± S.E.M. Q RT-PCR and ELISA data were analysed by the methods described previously in section 2.8.

5.3 Results

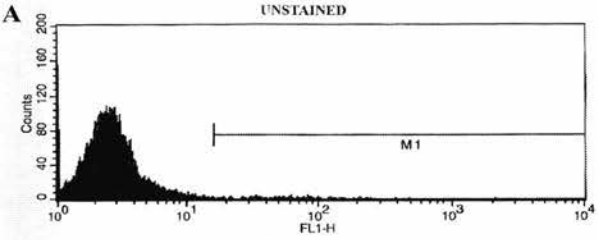
5.3.1 Purity of uNK cells isolated from 1st trimester decidua

Before embarking on a series of experiments to determine the impact of hCG on gene expression in uNK cells, the purity of isolated uNK cells had to be ascertained. FACS analysis confirmed purity of >90% from 1st trimester decidua (Figure 5.1, I) and > 95% from late secretory endometrium (Figure 5.1, II). This was further confirmed by IHF analysis of CD56 immunoexpression (Figure 5.2). In addition, uNK cells are reported to be highly granular and this was confirmed by morphological analysis of H&E stained cells (Figure 5.3).

Figure 5.1: FACS analysis of uNK cell purity. Panel I, 1st trimester MACS®-isolated uNK cells: unstained cells (A), isotype-matched negative control (B), CD56 FITC stained cells (C); the y axis is the total cell count and the x axis is the FITC value. The region represented by M1 indicates the number of cells that are positive for FITC. Graph D shows sorting gate applied to isolated 1st trimester uNK cells. Representative scans from 46 samples are shown; cells are typically > 90 % pure. Panel II, non-pregnant late secretory MACS®-isolated uNK cells: The isotype-matched negative control (A) incubated with the CD56 antibody (B); the y axis is the forward scatter plot and the x axis is the FITC value- the total level of fluorescence detected on the cells counted. Unstained cells (C), isotype-matched negative control (D), CD56 FITC stained cells (E). The region represented by M1 indicates the number of cells that are positive for FITC. This is ~ 1% in graph C (unstained) and D (isotype control) whereas in graph E (CD56 stained) cells are ~ 95 % pure.

I

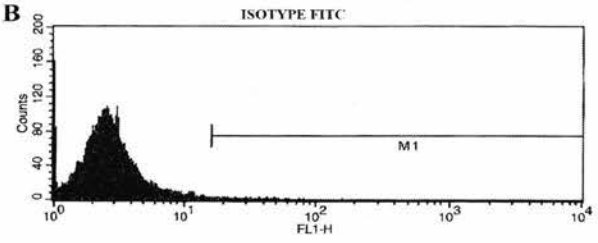
A



Sample ID: **Unstained** Gate: G1
Gated Events: 10000 Total Events: 12885

Marker	Events	% Gated	% Total	Geo Mean	CV	Median
All	10000	100.00	77.61	2.73	569.39	2.57
M1	135	0.35	1.05	56.78	210.44	53.76

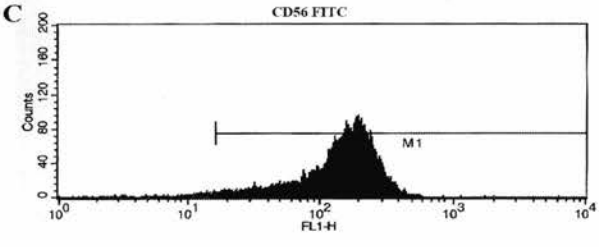
B



Sample ID: **Isotype FITC** Gate: G1
Gated Events: 9669 Total Events: 11676

Marker	Events	% Gated	% Total	Geo Mean	CV	Median
All	9669	100.00	82.81	2.75	112.40	2.59
M1	89	0.92	0.76	27.82	60.73	25.71

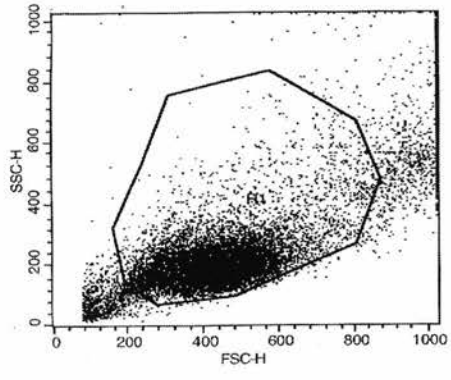
C



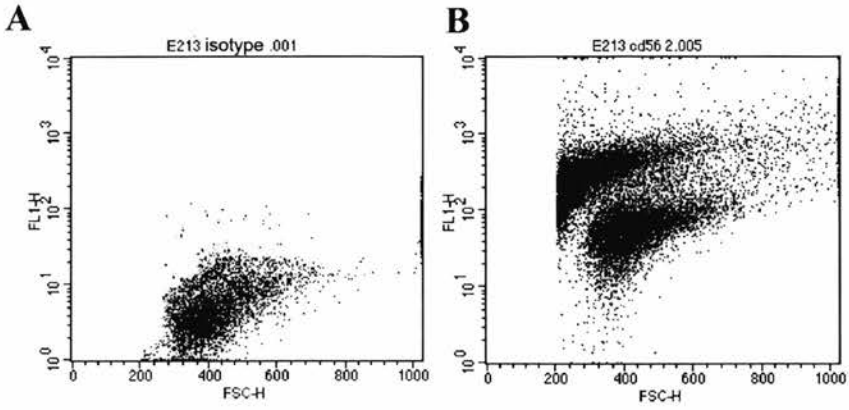
Sample ID: **CD56 FITC** Gate: G1
Gated Events: 9668 Total Events: 12375

Marker	Events	% Gated	% Total	Geo Mean	CV	Median
All	9668	100.00	78.13	130.24	89.74	158.20
M1	9441	97.34	76.29	139.12	87.64	159.63

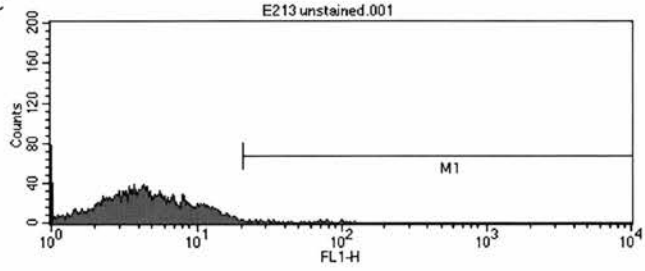
D



II



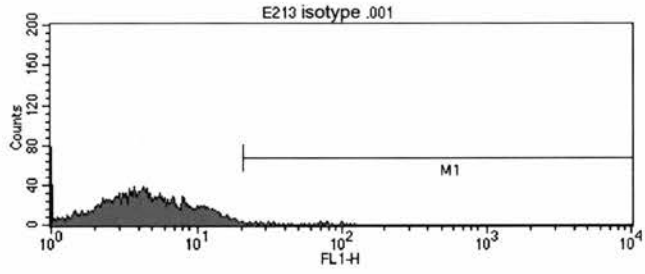
C



Sample ID: E213 unstained Gate: G1
Gated Events: 4818 Total Events: 10680

Marker	Events	% Gated	% Total	Geo Mean	CV	Median
All	4818	100.00	45.11	4.46	109.00	4.29
M1	48	1.00	0.45	41.56	59.87	33.08

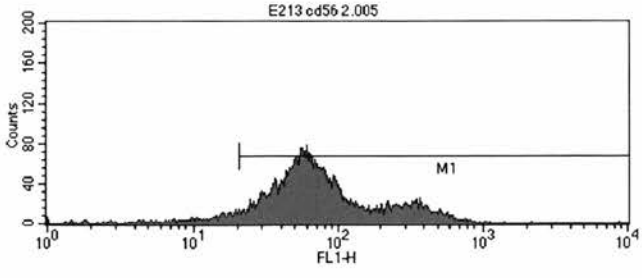
D



Sample ID: E213 isotype Gate: G1
Gated Events: 4818 Total Events: 10680

Marker	Events	% Gated	% Total	Geo Mean	CV	Median
All	4818	100.00	45.11	4.46	109.00	4.29
M1	48	1.29	0.45	41.56	59.87	33.08

E



Sample ID: E213 cd56 2 Gate: G1
Gated Events: 9270 Total Events: 21675

Marker	Events	% Gated	% Total	Geo Mean	CV	Median
All	9270	100.00	42.77	72.84	161.73	63.78
M1	8813	95.07	40.66	80.20	157.38	66.71

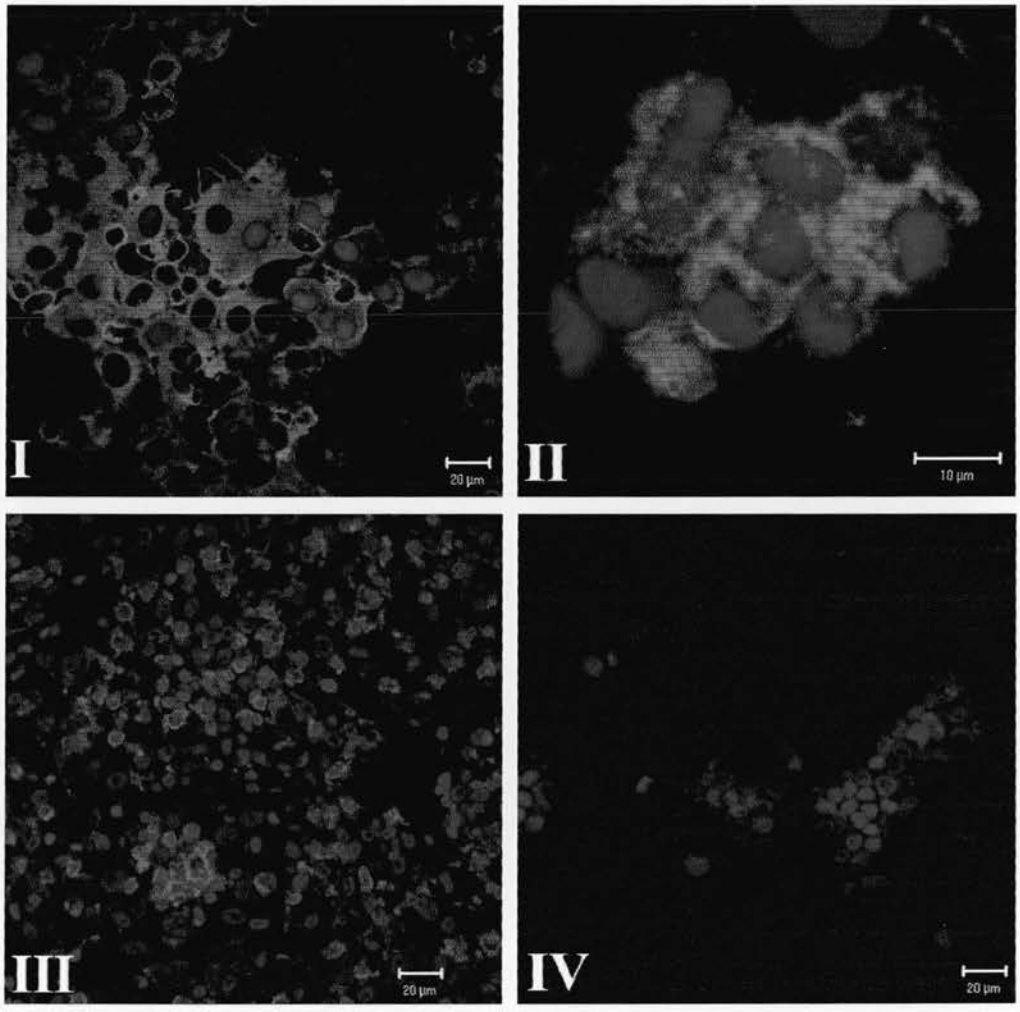


Figure 5.2: uNK cells immunostained for CD56. Red staining shows the localisation of CD56 antibodies; blue shows nuclear counterstain, To Pro. Panel I shows isolated uNK cells stained with mouse anti-human CD56 viewed at x 40 magnification with oil immersion. Panel II shows a cropped image of Panel I. Panel III shows a formalin-fixed section of 1st trimester decidua, included as a positive control. Panel IV shows the negative control. Note, not all cells stain positive for the nuclear counterstain as they were not permeabilised.

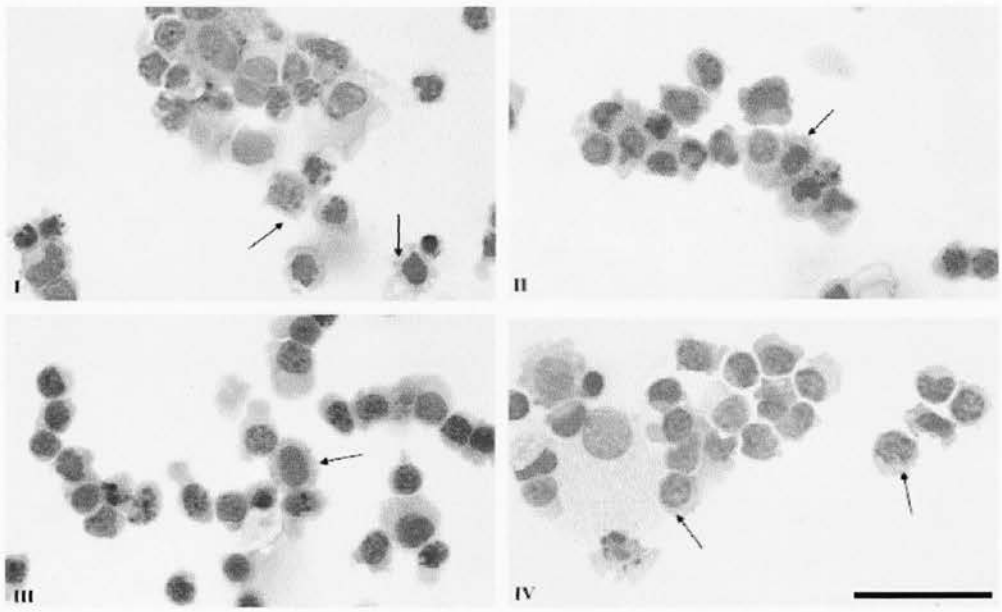


Figure 5.3: H&E staining of isolated NK cells from 1st trimester decidua. Panels I to IV, show representative uNK preparations from 1st trimester decidua preparations. Note the granularity of the cytoplasm, highlighted with black arrows, and the reniform, eccentric nucleus and short, cytoplasmic projections. Scale bars represent 100μm.

5.3.2 Expression of LH/hCG receptors in uNK cells isolated from 1st trimester decidua

To elucidate if the LH/hCG receptor was present in the uNK cells isolated from 1st trimester decidua, uNK samples were tested for the presence of LH/hCG receptor mRNA. Expression of LH/hCG receptor mRNA was only detected in the corpus luteum sample (positive control; kindly provided by Dr Colin Duncan) following semi-quantitative RT-PCR (Figure 5.4 Panel A, sample 8). No expression was observed in any of the samples of uNK cells isolated from 1st trimester decidua (Figure 5.4 Panel A, samples 1-7). Therefore any action by hCG will not be mediated by binding to the LH/hCG receptor. Panel B shows the same samples analysed using primers directed against GAPDH, which was used as an internal control for integrity of the RNA samples.

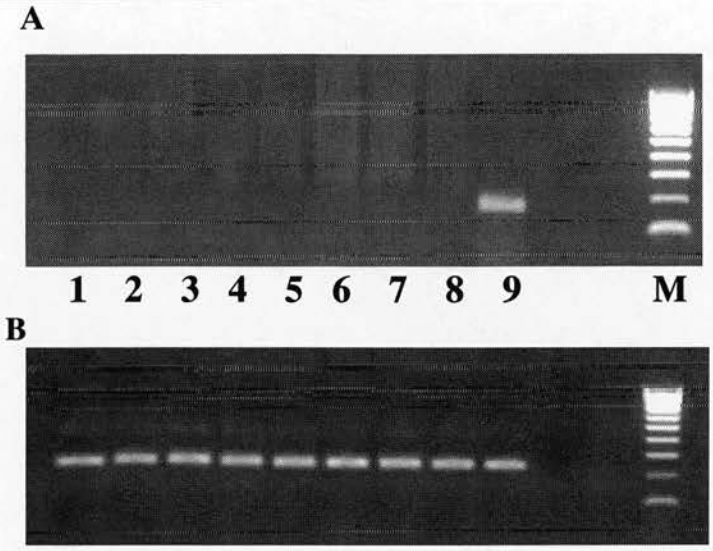


Figure 5.4: Expression of LH/hCG receptor (203 bp) was not detected in isolated uNK cells from 1st trimester decidua and late-secretory phase endometrium. A corpus luteum sample was included as a positive control. Samples were harvested and total cellular RNA was analysed by RT-PCR for the expression of LH (Panel A) and GAPDH (205bp) (Panel B), the housekeeping gene. Sample M represents DNA hyperladder. Samples 1 to 7 show uNK cells from 1st trimester decidua. Sample 8 shows uNK cells from non-pregnant late secretory phase endometrium. Sample 9 shows a corpus luteum sample.

5.3.3 hCG treatment increases uNK cell number *in vitro*

To elucidate if hCG has any effect on uNK cell proliferation, isolated uNK cells from 1st trimester decidua were cultured in the presence or absence of varying concentration of hCG, and analysed by light microscopy (Figure 5.5). Panel I represents cells exposed to no treatment, Panel II represents cells treated with 10 ng/ml hCG, Panel III shows cells treated with 100 ng/ml hCG, Panel IV shows cells treated with 1000 ng/ml hCG and Panel V represents cells treated with 10000 ng/ml hCG. All samples were treated for 2 h. No increase in cell number is observed with 10 ng/ml or 100 ng/ml hCG treatment (Figure 5.5, Panels II and III) when compared to control cells (Figure 5.5, Panel I). However, an increase is observed with 1000 ng/ml and 10000 ng/ml hCG treatment (Figure 5.5, Panels IV and V). A photograph from representative tissue culture of 12 decidual samples is shown (Figure 5.5). This

method is non-quantitative and therefore further studies to quantify the increase in proliferation are warranted.

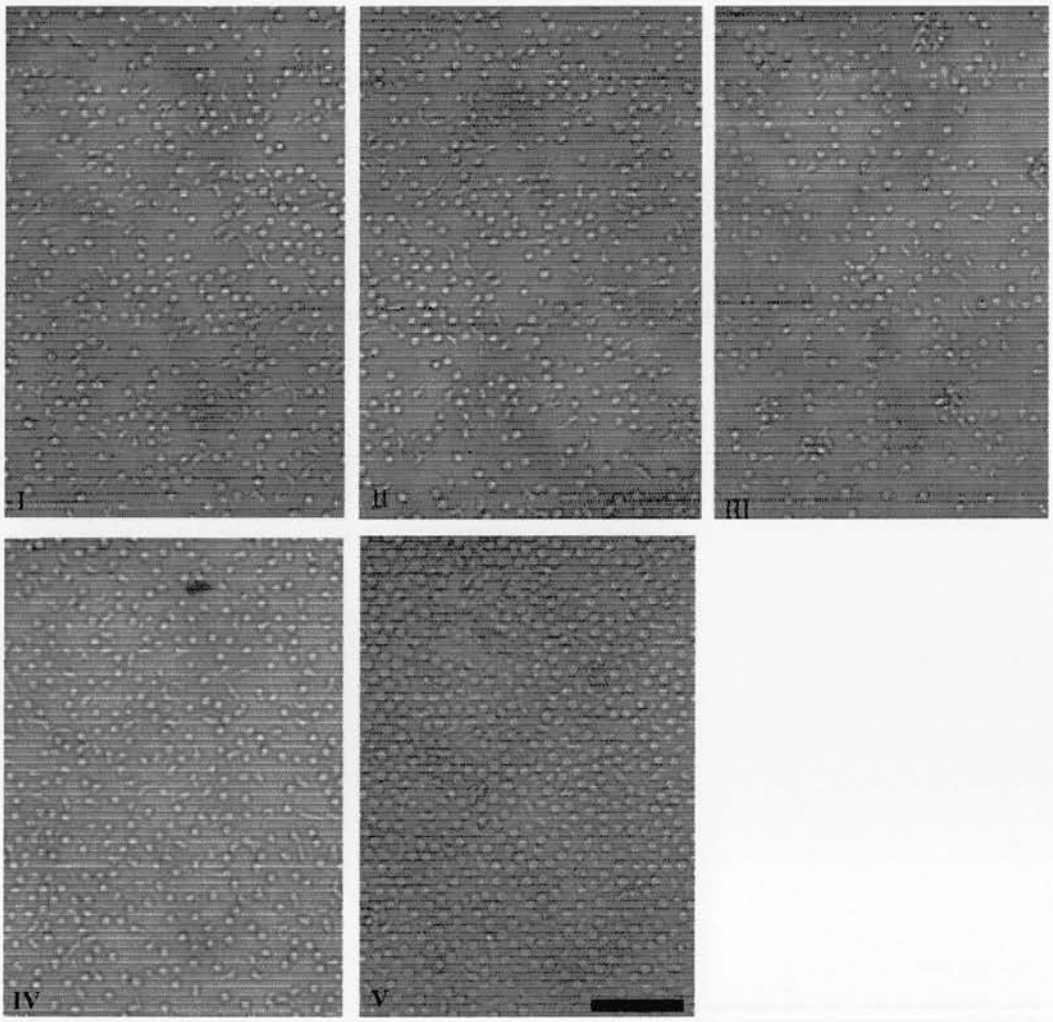


Figure 5.5: Photograph of uNK cells from 1st trimester decidua in culture, treated with increasing concentrations of hCG for 2h. Panel I shows no treatment, Panel II shows 10 ng/ml hCG, Panel III shows 100 ng/ml hCG, Panel IV shows 1000 ng/ml hCG and Panel V shows 10000 ng/ml hCG. Photograph from representative tissue culture of 12 decidual biopsies. Scale bars represent 50μm.

5.3.4 hCG-mediated effect on the production of uNK-associated cytokines

To determine if hCG could affect cytokine release of uNK cells, the production of several cytokines associated with uNK cells were measured after treatment with hCG. No change in expression of TNFα (Figure 5.6, Panel A), IL-10 (Figure 5.6, Panel B), and IFNγ (Figure 5.6, Panel C) mRNAs and TNFα protein production (Figure 5.7, Panel A) was observed with hCG treatment at any concentration, n = 8 uNK 1st trimester samples. In contrast, IL-10 protein release was significantly increased with 10 ng/ml hCG treatment (Figure 5.7, Panel B, $p<0.05$), uNK 1st trimester samples). No effect was seen with any other concentration used (Figure 5.7, Panel B). IFNγ protein release was significantly inhibited with treatment of 10 ng/ml and 100 ng/ml hCG after 2 h (Figure 5.7, Panel C, $p<0.01$, n= 8 uNK 1st trimester samples). Treatments with 1000 ng/ml and 10000 ng/ml hCG were without significant effect, but displayed a mild trend towards augmentation.

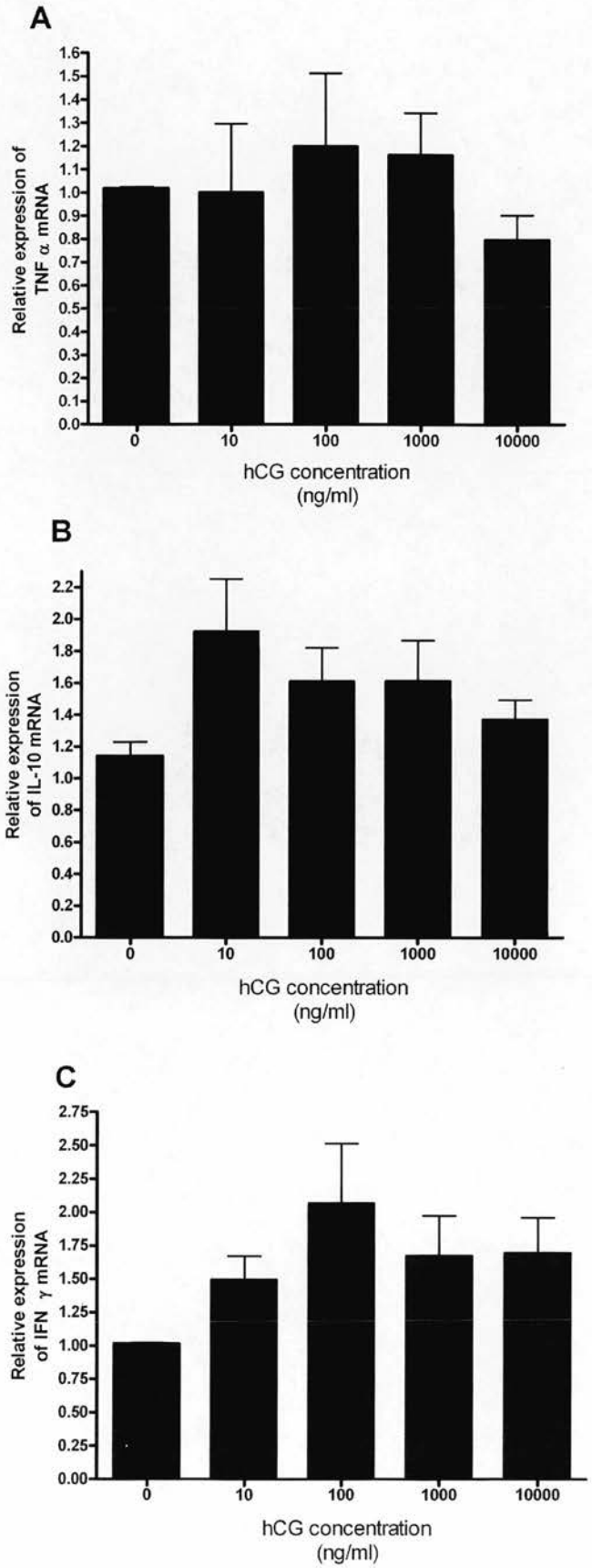


Figure 5.6: TNFα (Panel A), IL-10 (Panel B) and IFNγ (Panel C) mRNA expression in uNK cells incubated with increasing concentrations of hCG. No significant effect was observed in any cytokine with all concentrations of hCG treatment, 2h. n = 8 uNK 1st trimester samples.

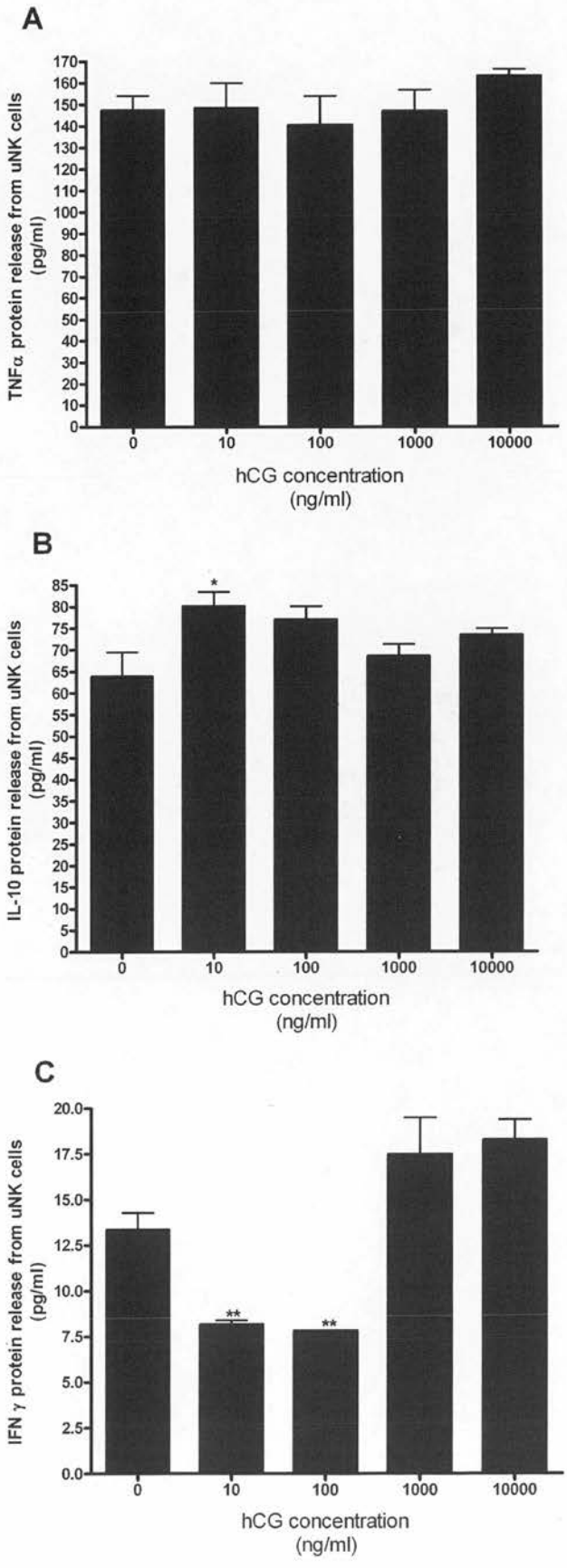


Figure 5.7: IL-10 (Panel A), TNFα (Panel B) and IFNγ (Panel C) release from uNK cells incubated with increasing concentrations of hCG. Treatment with hCG, 2h, failed to induce a response in TNFα protein release, n = 8 isolated uNK samples. The lower detection limit of the ELISA was 31.2 pg/ml. Treatment with 10 ng/ml hCG, 2h, evoked a significant increase in IL-10 protein release from uNK cells (Panel B, $p<0.05$, n = 8 isolated uNK samples). The lower detection limit of the ELISA was 7.8 pg/ml. Treatment with 10 ng/ml and 100 ng/ml hCG, 2 h, evoked a significant reduction in IFNγ protein release from uNK cells (Panel C, $p<0.01$, n = 8 isolated uNK samples). Treatment with 10 ng/ml and 100 ng/ml hCG show no significant effect. The lower detection limit of the ELISA was 7.8 pg/ml.

5.3.5 Expression of MMR on uNK and PBNK cells

Expression of MMR mRNA was detected in both 1st trimester uNK cells and in the decidual sample (Figure 5.8 samples 1 to 6 and 8, panel A). MMR mRNA was low in non-pregnant secretory phase uNK cells (Figure 5.8, panel A, sample 7) in the semi-quantitative PCR. Panel B shows the same samples analysed using primers directed against for GAPDH, which was used as an internal control for integrity of the RNA samples. This was further corroborated with quantitative RT-PCR (TaqMan) conducted on the same samples (Figure 5.9). Protein expression was identified with IHF and FACS analysis. Isolated 1st trimester uNK cells were immunoprobed for MMR (Figure 5.10). Although MMR is a cell surface receptor it is internalised upon glycosylation, therefore the cells were permeabilised prior to immunostaining. The staining pattern of MMR is not uniform but would appear to be quite clustered. Isolated 1st trimester uNK cells were stained for MMR, CD56 and both in combination and analysed by FACS to identify the population of uNK cells positive for MMR. Results are presented in Figure 5.11; panel A shows cells without staining, Panels B and C show cells stained with the mouse isotype control FITC and PE respectively, Panel D shows cells stained with CD56, Panel E shows cells stained with CD206 (MMR) and Panel F shows cells stained with both CD56 and CD206. As reported previously, isolated cells are >90 % pure (Figure 5.11, Panel E, lower right quadrant (LR)). Not all CD56 positive cells stain positive for CD206, indeed only approximately 40% of these cells are positive for CD206 (Figure 5.11, Panel E, upper left quadrant (UL)). ~35 % of CD206 positive cells also stain positive for CD56 (Figure 5.12, Panel F, upper right quadrant (UR)). Anomalies in percentages may be attributable to either antibody masking antigenic sites or preventing binding of the other antibody to the cells. Neither isotype control was seen to induce changes (Figure 5.11, Panels B and C).

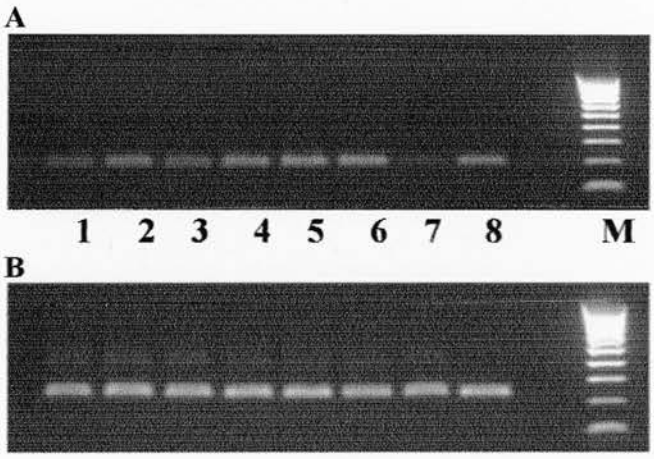


Figure 5.8: Expression of MMR mRNA (203bp) in isolated uNK cells from 1st trimester decidua and late-secretory phase endometrium. A decidual sample was included as a positive control. Samples were harvested and total cellular RNA probed for the expression of MMR (Panel A) and GAPDH (205bp) (Panel B), the housekeeping gene. Sample M represents DNA hyperladder. Samples 1 to 6 show uNK cells from 1st trimester decidua. Sample 7 shows uNK cells from non-pregnant late secretory phase endometrium. Sample 8 shows a decidual sample.

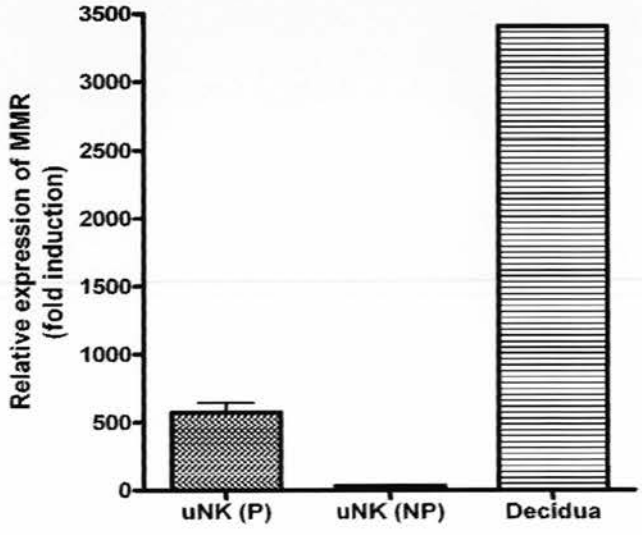


Figure 5.9: Preliminary data on the relative expression of MMR gene in uNK cells isolated from 1st trimester decidua (P) or from late secretory non-pregnant endometrium (NP). A decidual sample was included as a positive sample. MMR mRNA was analysed by Q-RT-PCR. Results are ± SEM n = 6 uNK (P) samples, n = 1 uNK (NP) sample and n = 1 decidual sample. All samples were run in triplicate.

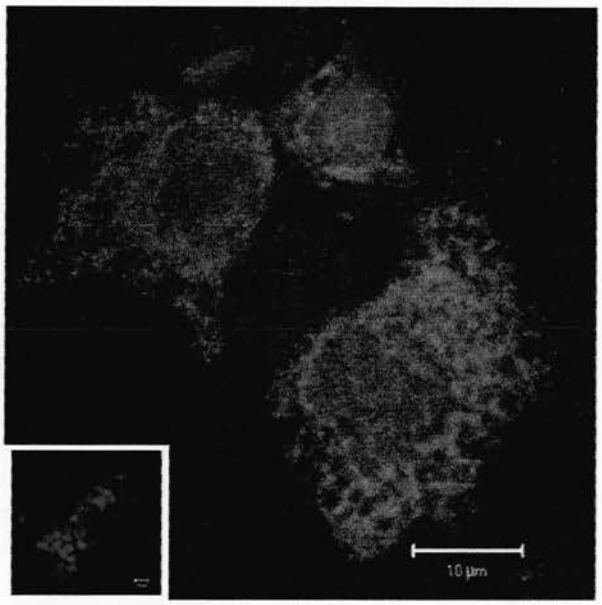
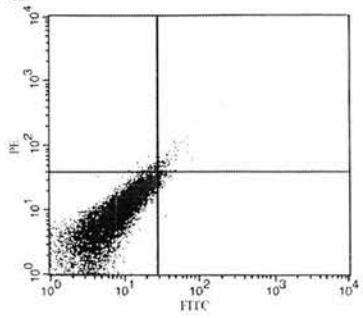


Figure 5.10: uNK cells immunoprobed for MMR. Green shows MMR staining; blue shows nuclear counterstain, To Pro. Insert shows negative control.

Figure 5.11: FACS analysis of uNK cell surface antigen expression. On graphs A to F, axis y is the PE value and axis x is the FITC value; Panel A shows unstained cells (B), isotype-matched negative controls for FITC and PE respectively (B and C), 1st trimester MACS®–isolated uNK cell sample stained for CD56 (D) and MMR (E), respectively, Graph F shows representative co-expression analysis of CD56 and MMR. One representative data set is shown of nine experiments performed. FITC, fluorescein isothiocyanate; PE, phycoerythrin; UL, upper left quadrant; UR, upper right quadrant; LL, lower left quadrant; LR, lower right quadrant.

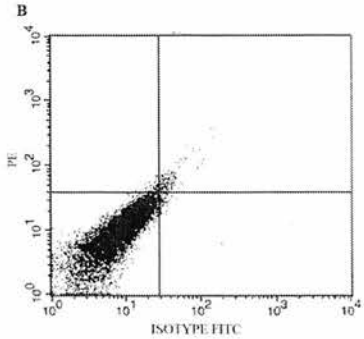
A



Sample ID: Unstained
Gate: G1
Gated Events: 10000
Total Events: 13150

Quad	Events	% Gated	% Total
UL	71	0.71	0.54
UR	96	0.96	0.73
LL	9758	97.58	74.21
LR	75	0.75	0.57

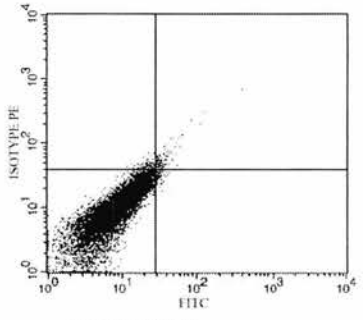
B



Sample ID: Isotype FITC
Gate: G1
Gated Events: 10000
Total Events: 14560

Quad	Events	% Gated	% Total
UL	71	0.71	0.49
UR	150	1.50	1.03
LL	9693	96.93	66.57
LR	86	0.86	0.59

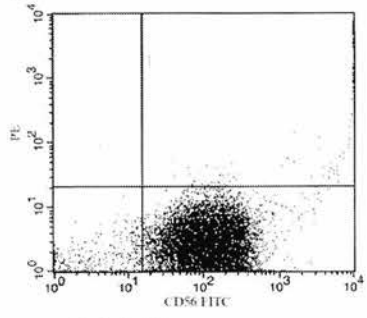
C



Sample ID: Isotype PE
Gate: G1
Gated Events: 10000
Total Events: 14048

Quad	Events	% Gated	% Total
UL	84	0.84	0.60
UR	103	1.03	0.73
LL	9732	97.32	69.28
LR	81	0.81	0.58

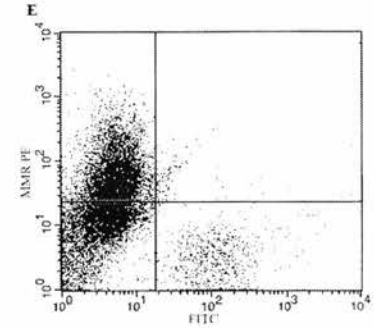
D



Sample ID: CD56
Gate: G1
Gated Events: 10000
Total Events: 25572

Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	116	1.16	0.45
LL	408	4.08	1.60
LR	9476	94.76	37.06

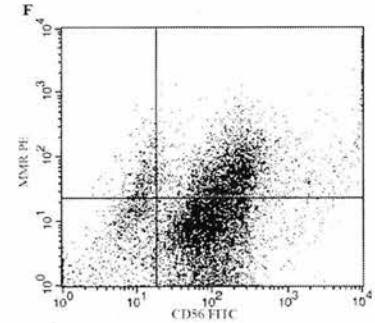
E



Sample ID: MMR
Gate: G1
Gated Events: 10000
Total Events: 30520

Quad	Events	% Gated	% Total
UL	4648	46.48	15.23
UR	121	1.21	0.40
LL	4559	45.59	14.94
LR	672	6.72	2.20

F



Sample ID: CD56/MMR
Gate: G1
Gated Events: 10000
Total Events: 31210

Quad	Events	% Gated	% Total
UL	328	3.28	1.05
UR	3066	30.66	9.82
LL	906	9.06	2.90
LR	5700	57.00	18.26

5.3.6 hCG colocalises with MMR in uNK cells isolated from 1st trimester decidua

To elucidate if hCG may bind to MMR, uNK cells treated with hCG were immunoprobed for both hCG and MMR (Figure 5.12). Panels I, II and III shows 1st trimester uNK cells stained with rabbit anti-human hCG (red) and mouse anti-human MMR (green). Panels IV, V and VI show cropped images of the samples in the panels above. Panel VII shows placenta stained for hCG as a positive control. Panels VII and IX shows negative controls, placenta and uNK cells respectively. As observed with FACS analysis, less than half of the isolated 1st trimester uNK cells stained positive for MMR, localised predominantly to the cell surface (Figure 5.12, panels I, II, III). All cells stained postitive for hCG, again predominantly in a cell surface location (Panels I to VI). Areas of colocalisation were observed and appear as yellow in the images.

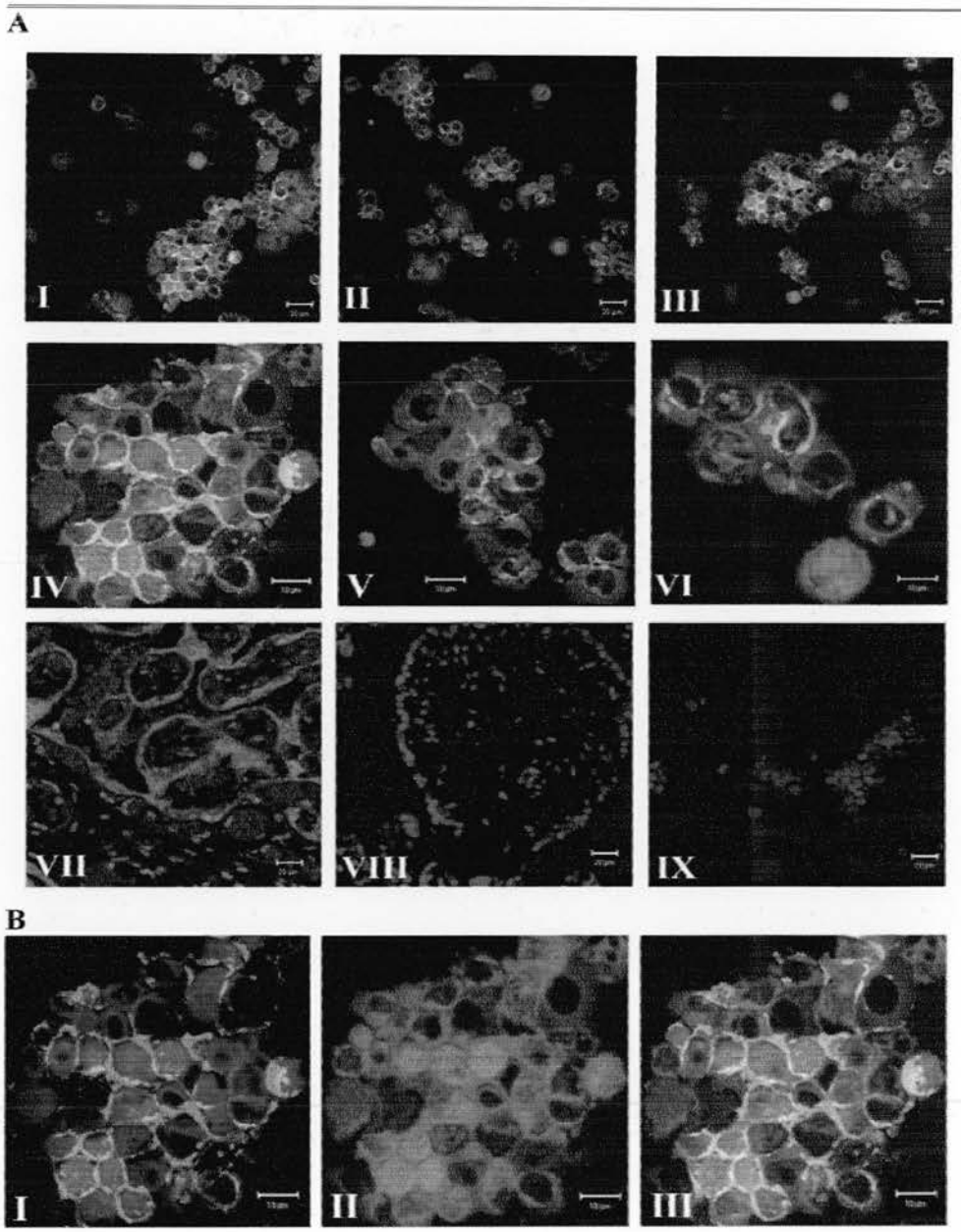


Figure 5.12: Staining of uNK cells with MMR staining illustrated by green and hCG staining by red. Yellow staining shows areas of colocalisation. Blue shows nuclear counterstain, To Pro. Panel A: panel I, II and III shows uNK cells at x 40 magnification with oil immersion. Panels IV, V and VI show cropped image of panels I, II and III respectively. Panel VII shows a formalin fixed placenta section. Panels VIII and IX show negative controls, both uNK cells and placenta, respectively. Panel B: shows split image of panel IV; panel I shows MMR alone, panel II shows hCG alone, panel III shows panels I and II superimposed.

5.4 Discussion

This study has demonstrated that low doses of hCG, <1000 ng/ml, promoted IL-10, and inhibited IFNγ protein release by uNK cells, isolated by MACS® separation from 1st trimester decidua (Figure 5.7, Panels B and C). Previous studies showed that hCG enhanced TNFα and IFNγ protein production by PBMC (Schafer, Pauli et al. 1992; Yousefi, Karamlou et al. 1993). The results in this study are in contrast to the previous findings, and demonstrate that, in uNK cells; hCG inhibits protein release of IFNγ and is without effect on TNFα mRNA or protein. The inhibitory effect of hCG on IFNγ and stimulatory effect on IL-10 protein release was not observed at mRNA level. The observed hCG responses may be cell-specific. Treatment with 10,000 ng/ml hCG also appeared to increase uNK cell number (Figure 5.5, n = 12 decidual samples). However, as no quantitative analysis was conducted, hCG-induced cell proliferation cannot be conclusively stated. Furthermore, to ensure that the population of cells undergoing apparent cellular proliferation is $CD56^{Bright}$ uNK cells, FACS analysis, determining cell surface expression of uNK cell-specific markers must be undertaken.

hCG can propagate its hormonal signal to target cells through the LH/HCG receptor, which was originally reported to be expressed on ovarian granulosa cells, theca interna cells, and luteal cells (Acevedo 2002). Several lines of evidence have demonstrated that the LH/hCG receptor is also expressed in the endothelium and myometrium (Reshef, Lei et al. 1990; Lei, Reshef et al. 1992; Kornyei, Lei et al. 1993). A previous study reported that mRNA of the LH/HCG receptor was detected in T lymphocytes derived from the peripheral blood of pregnant women (Lin, Lojun et al. 1995). In addition, the LH/hCG receptor has been localised to macrophages from term placenta and late secretory phase non-pregnant endometrium (Zhang, Rao Ch et al. 2003). In the present study, uNK cells, isolated by MACS® separation from 1st trimester decidua, were shown to respond to hCG by releasing IL-10 protein (Figure 5.7, Panel B, $p<0.05$, n = 8 decidual samples), and inhibiting IFNγ protein release (Figure 5.7, Panel C, $p<0.01$, n = 8 decidual samples). However, expression of LH/hCG receptor mRNA was not detected in $CD56^{Bright}$ uNK cells from 1st

trimester decidua and late secretory non-pregnant endometrium by RT-PCR (Figure 5.4). As the uNK cells in this study were cultured alone, and any hCG-mediated responses would be a result of direct interaction with the uNK cell, the findings herein indicate a discrepancy between hCG-binding and LH/hCG receptor distribution. This is in agreement with studies reporting no LH/hCG receptor expression in PBNK cells (Sulke, Jones et al. 1985; van den Heuvel, Peralta et al. 2005) and PBMN cells, stimulated by exogenous hCG (Kosaka, Fujiwara et al. 2002),

Furthermore, a recent study has demonstrated that addition of mannose significantly suppressed hCG-stimulated IL-8 production and interfered with the binding of hCG to CD14-positive monocytes (Kosaka, Fujiwara et al. 2002). The authors proposed that cell surface lectins may play a role in the hCG-signalling pathway; however they failed to detect MMR mRNA in their cell preparation (Kosaka, Fujiwara et al. 2002). Recently, pituitary hormones such as LH and TSH were reported to interact with MMR (Fiete, Beranek et al. 1998; Simpson, Hitchen et al. 1999) and it was proposed that immune cells are involved in the clearance of these hormones (Simpson, Hitchen et al. 1999). This receptor recognises glycoconjugates terminating in mannose, fucose, or N-acetylglucosamine in a Ca^{2+}-dependent manner via C-type carbohydrate-recognition domains (Taylor 2001). Therefore, we examined if the MMR was present on uNK cells, both from non-pregnant endometrium and 1st trimester decidua, and whether uNK cells treated with hCG would demonstrate hCG-binding and if this would colocalise with MMR. MMR mRNA and protein was detected in uNK cells isolated from 1st trimester decidua (Figure 5.8, samples 1-6 and Figure 5.10) The protein distribution on the uNK cell surface appeared to be quite clustered (Figure 5.10), although this may represent the constant recycling of the MMR between the cell surface and the cytoplasm (East and Isacke 2002). MMR mRNA expression in uNK cells isolated from a non-pregnant late secretory sample was low in comparison to uNK cells derived from decidua, as analysed by RT-PCR. This was further corroborated with quantitative analysis (Figure 5.9), however, such comparisons should proceed with caution as these are very preliminary data and further analysis of many more uNK samples isolated from non-pregnant

endometrium is required. Moreover, both RT-PCR and Q-RT-PCR analysis demonstrated markedly higher expression of MMR mRNA in decidua than in isolated uNK cells, this may be explained by MMR expression on cells in decidua other than the uNK cell, e.g. macrophages (Shepherd, Tarnowski et al. 1991; Taylor, Gordon et al. 2005). FACS analysis demonstrated that not all uNK cells express MMR on the cell surface, indeed only ~ 40 % of the uNK cells stain positive (Figure 5.11, Panel E, UL). Again, this may reflect shuttling of the receptor as opposed to uNK cell distribution of MMR. However, this may indicate the presence of a sub-population of uNK cells that express MMR in addition to the classic uNK cell marker $CD56^{Bright}$. This distribution pattern was also observed with IHF studies in which not every uNK cell stained positive for MMR (Figure 5.12, Panel A (I, II and III)). Interestingly, after treatment with hCG, most antigenic sites positive for MMR showed colocalisation with hCG (Figure 5.12, Panel A (IV, V and VI), Panel B). Conversely, several antigenic sites positive for hCG did not exhibit colocalisation. In the absence of an LH/hCG receptor, additional binding sites for hCG may exist and it is notable that other C-type lectins have been identified on immune cells (Ebner, Ehammer et al. 2004). For example proliferating antigen-presenting cells expressing DC-SIGN (CD209) has been identified in the decidua of early human pregnancy (Kammerer, Eggert et al. 2003).

A very recent study has demonstrated that glycosylation, terminal sialylation in particular, differentially influences the lectin activities of MMR, allowing oligomerisation of the receptor, resulting in multiple presentations of CR domains (Su, Bakker et al. 2005). The authors also propose that this glycosylated-regulation of MMR may facilitate binding of two sulfated hCG glycans, into two neighbouring CR domain binding pockets (Su, Bakker et al. 2005), providing a potential mechanism for the interaction between glycosylated hormones and MMR. Additionally, Kovalevskaya *et al* (Kovalevskaya, Birken et al. 2002) reported that a hyperglycosylated form of hCG arises very early in pregnancy and is rapidly replaced by less glycosylated isoforms, showing that hCG glycoforms dynamically change as pregnancy progresses. This finding has been suggested to provide a rationale for the

proposed maternal recognition of the embryo during the earliest stages of pregnancy in that the embryo may transmit the information about its presence to maternal immune systems via highly glycosylated hCG, which mimics products produced in bacterial infections (Kosaka, Fujiwara et al. 2002). Therefore, the physiological roles of hCG not only in the endocrine system, but also in the immune system should be investigated further with respect to aspects other than the so-called LH/HCG receptor.

In conclusion, the present study demonstrated that uNK cells derived from 1st trimester decidua are able to respond to hCG at concentrations < 1000 ng/ml by enhancing their production of IL-10 and reducing their production of IFNγ. This study also demonstrated that a signalling pathway different from the LH/hCG receptor system are responsible for this effect of hCG on uNK cells. In addition, MMR was localised to uNK cells and IHF analysis showed areas of MMR and hCG colocalisation, suggesting a potential mechanism by which hCG may bind to uNK cells. These observations now require further functional studies to elucidate if a potential LH/hCG – MMR interaction can facilitate downstream signalling and induce proliferation and/or migration, providing an alternative mechanism for the expansion of uNK cells observed during the late-secretory phase of the non-pregnant menstrual cycle and the 1st trimester of pregnancy. These findings suggest that an immune-endocrine network involving hCG and uNK cells exists and may play an important role in regulation of the menstrual cycle and events of early pregnancy.

Chapter 6

6 General Discussion

6.1 Introduction

Decidualisation of the endometrial stromal compartment is essential for implantation of the conceptus but, in the absence of pregnancy, loss of the decidual phenotype is functionally linked to menstrual shedding of the endometrium (Bell 1990). Decidualisation is governed by the steroid hormone progesterone acting via its nuclear receptor (Huang, Tseng et al. 1987; Zhu, Huang et al. 1990; Tseng, Gao et al. 1992; Mizuno, Tanaka et al. 1998; Brosens, Hayashi et al. 1999), but at a paracrine level may be regulated by a myriad of cytokines and growth factors, including TGFβ1 (Jones, Stoikos et al. 2006). The Wnt-signalling pathway antagonist, Dickkopf (DKK) has also been implicated in the process of decidualisation as DKK expression is localised to the stroma and increased in mid-secretory phase endometrium (Tulac, Nayak et al. 2003; Giudice 2004). Additionally, DKK is reported to be progesterone-dependent (Tulac, Overgaard et al. 2006). TGFβ1 is abundantly expressed in both endometrial epithelial and stromal cells and is thought to play a critical role in cyclic tissue remodelling and inflammatory events associated with menstruation (Bruner, Rodgers et al. 1995; Ulloa, Creemers et al. 2001). TGFβ1 mediates its actions predominantly via the SMAD signalling pathway (Graff, Bansal et al. 1996; Macias-Silva, Abdollah et al. 1996; Nakao, Imamura et al. 1997; Massague and Wotton 2000; Shi and Massague 2003). However when the current studies were initiated it remained to be determined whether TGFβ1 affects either PR or DKK to regulate the decidual process.

Decidualisation is also associated with a unique immune environment, characterised by the presence of large numbers of uterine-specific natural killer cells (uNK)

(Bulmer, Lunny et al. 1988). uNK cells increase in number in secretory phase endometrial stroma, implying regulation by progesterone on their expansion in number (King 2000). However, they lack the nuclear progesterone receptor (Henderson, Saunders et al. 2003) and growth and differentiation may therefore depend on interactions with endometrial stromal cells (ESCs) and regulation by other cytokines (Trundley and Moffett 2004) or may involve other hormones such as luteinising hormone (LH) and human chorionic gonadotrophin (hCG).

6.2 Findings of this thesis

These studies described in this thesis set out to investigate the role of TGFβ1 in modulating the regulators of decidualisation and the resulting production of decidualisation marker proteins, in an attempt to achieve better understanding of how the process of decidualisation may be regulated *in vivo*. Additionally, the studies herein have sought to characterise better the phenotypically unique uNK cell, whose numbers expand in the mid to late secretory phase of the cycle in parallel with decidualisation of the stroma.

In Chapter 3, studies focused on the impact of TGFβ1 on gene expression in non-decidualised and decidualised stromal cells. The studies in this chapter were performed using primary human ESCs, either non-differentiated or treated with 8-Br-cAMP and MPA to induce a decidualised phenotype. Initially, studies were designed to determine the effect of TGFβ1 treatment on SMAα mRNA expression. The studies presented herein demonstrate that TGFβ1 treatment significantly augments SMAα mRNA expression in both non-decidualised (Figure 3.7 and 6.13) and decidualised ESCs (Figure 3.8 and 6.13). Previous papers have reported that human stromal cells, which had been decidualised *in vivo* in the presence of a blastocyst, expressed SMAα and displayed ultrastructural similarities with myofibroblasts (Oliver, Montes et al. 1999). Oliver *et al* also reported that SMAα could be detected by FACS analysis in decidual stromal cells, decidualised *in vitro* by treatment with progesterone (Oliver, Montes et al. 1999). 1st trimester decidua sections were also immunoprobed for αSMA, which was localised to the perivascular cells (Oliver,

Montes et al. 1999). The stromal cells in the 1st trimester tissues used in the study by Oliver *et al* would have been exposed to progesterone for at least 8 or 9 weeks and should have been fully decidualised *in vivo* (Oliver, Montes et al. 1999). Previous work in our laboratory has demonstrated a similar perivascular immunolocalisation of SMAα in mid-secretory phase endometrium (Rodney Kelly and Sheila MacPherson, unpublished observations). TGFβ1, which has previously been demonstrated to be an inducer of a myofibroblast phenotype (Desmouliere, Geinoz et al. 1993; Jester, Barry-Lane et al. 1996; Serini, Bochaton-Piallat et al. 1998; Grotendorst, Rahmanie et al. 2004; Lewis, Lygoe et al. 2004; Shephard, Martin et al. 2004), has also been shown to induce contractility of DSC (Kimatrai, Oliver et al. 2003). The studies described in Chapter 3 demonstrated that TGFβ1 upregulates SMAα mRNA expression in both non-decidualised and decidualised ESC in agreement with the previous studies. In the current study, treatment with TGFβ1 induced a myofibroblast phenotype (induction of SMAα) in both non-decidualised and decidualised ESCs (Figures 3.7 and 3.8). In contrast, Oliver *et al* have claimed that SMAα is only present in DSCs when cultured with progesterone for 14 d (Oliver, Montes et al. 1999). The stromal cells, in which TGFβ1 induces a myofibroblast phenotype *in vitro,* are the only endometrial cell type that retains their PR throughout the menstrual cycle, including during decidualisation (Wang, Critchley et al. 1998).

As progesterone is the hormone thought to be responsible for the process of decidualisation (Brosens, Hayashi et al. 1999) and TGFβ1 has been detected in its active form during the late-secretory and pre-menstrual phase of the cycle (Casslen, Sandberg et al. 1998) further studies presented in this thesis concentrated on a possible TGFβ1-mediated regulation of PR expression. We report that TGFβ1 induced a rapid upregulation of PR mRNA expression in both non-decidualised (Figure 3.10) and decidualised (Figure 3.12) ESCs followed by a subsequent downregulation (Figures 3.10 and 3.12), which was mirrored at a mature protein level (Figures 3.11, 3.13, 3.14, and 6.3). However, Western blot analysis of the reduction in PR protein (Figure 3.14) was not convincing and requires further

experimentation with an increased number of samples and densometric analysis. This inhibitory response was also observed in the PR positive breast cancer T47D cell line (Figures 3.12 and 3.13). The initial TGFβ1-induced upregulation of PR may be mediated by the non-genomic PRs, however this was not analysed further. As no functional role has been identified for the non-genomic receptors it is impossible to identify downstream target genes which could be used to assess any impact of TGFβ1 on these putative membrane-bound receptors. The downregulation of PR in both ESCs and T47D cells would suggest that the effect of TGFβ1 is perhaps not stromal cell-specific but PR-specific. Furthermore, by transiently transfecting ESCs with a PRE or an ERE linked to luciferase, as a reporter gene, we could demonstrate that the TGFβ1-induced downregulation of PR was not due to TGFβ1-signalling preventing ER-mediated PR gene transcription (Figure 3.15). This result suggested that the observed TGFβ1-induced downregulation of PR was without effect on gene transcription, but could perhaps be attributed to TGFβ1-induced alteration of RNA stability, which may reduce the half-life of the mRNA and subsequently impact mRNA expression and protein translation. Another possibility is that TGFβ1 may be acting on other sites of the PR promoter, e.g. SMAD binding sites, which in turn may impact PR gene transcription via recruitment of co-activators or co-repressors, or TGFβ1 may be interacting directly with co-regulators to impede PR binding to its PRE, thus suppressing PR gene transcription. Further experimentation transfecting ESCs with promoter-reporter constructs containing SMAD binding sites in addition to a PRE or ERE may elucidate a mechanism by which TGFβ1 impacts PR expression.

Previous studies have reported that Wnt family members are expressed in human endometrium throughout the menstrual cycle (Tulac, Nayak et al. 2003). The authors reported no significant menstrual cycle dependence of the Wnt ligands (except Wnt-3), receptors, or downstream effectors (Tulac, Nayak et al. 2003). The authors reported that Wnt-3 was significantly increased in proliferative compared with secretory phase endometrium in accordance with data from Hou *et al* who report that canonical Wnt signalling is critical to oestrogen-mediated uterine growth (Tulac,

Nayak et al. 2003; Hou, Tan et al. 2004). Tulac *et al* also reported a significant up-regulation of DKK in the secretory, compared with the proliferative phase, and that expression was restricted to the stroma (Tulac, Nayak et al. 2003; Giudice 2004). More recent findings have elucidated that DKK mRNA synthesis and protein expression is upregulated in human ESCs, decidualised *in vitro*, and that the response is progesterone specific and independent of cAMP and oestradiol (Tulac, Overgaard et al. 2006). However, analysis of the DKK promoter region did not reveal progesterone response element consensus sequences (Tulac, Overgaard et al. 2006). The studies presented herein are in agreement with those of Tulac *et al* and demonstrate induction of DKK protein release associated with decidualisation *in vitro* (Figure 3.17, 3.19, 3.21, and 6.3). Additionally, to our knowledge we report for the first time that TGFβ1 suppresses DKK mRNA expression and protein release in a TGFβ1-specific manner and that this downregulation precedes that of PR mRNA downregulation (Figure 3.16 – 3.19). If DKK is indeed involved in decidualisation then perhaps this initial inhibition of DKK induces Wnt-signalling and heralds the onset of TGFβ1-induced abrogation of the decidualised phenotype in the ESC during the normal menstrual cycle. As with ERE, TGFβ1 treatment did not impact the reporter gene activation of the PRE/ARE or GRE, which share an identical sequence (Figure 3.15). However, as no PRE has as yet been identified on the DKK promoter, any potential TGFβ1-induced effect would be redundant with regards to DKK mRNA transcription. To date, no evidence has been provided for the presence or absence of an ARE or GRE on the DKK promoter. Interestingly, a recent publication by Goldman et al has provided a possible mechanism for PRE-independent progesterone mediated responses (Goldman and Shalev 2006). Goldman proposes that in addition to the direct transcriptional activation through binding with its cognate DNA response element, PR is capable of transcriptional activation interacting with other classes of transcription factors on their cognate binding site, e.g. CREB-binding protein and/or SP-1 (Goldman and Shalev 2006). The authors suggest that in such cases where PR acts in a PRE-independent manner, that progesterone acts as a co-activator or co-repressor (Goldman and Shalev 2006). Although this proposed mechanism has not yet been conclusively proven, it does suggest a potentially novel process by which progesterone regulates DKK.

Although we report that TGFβ1 can induce SMAD phosphorylation consistent with activation of its signalling pathway in the cells maintained *in vitro* in the studies presented in this thesis (Figures 3.22 –3.23), no conclusive findings can be drawn on whether the downregulation of PR and DKK are SMAD-regulated. Identification of the pathways regulating the suppression of PR and DKK will be important for evaluating the TGFβ1-induced expression of PIAS γ, which we have observed in agreement with previous published data from Imoto *et al* (Imoto, Sugiyama et al. 2003). As PIAS γ has been reported to inhibit SMAD transcriptional activity, this TGFβ1-induced expression of PIAS γ (Figure 3.14) may provide a mechanism by which TGFβ1 inhibits the expression of PR and perhaps also DKK (summarised in Figure 6.1).

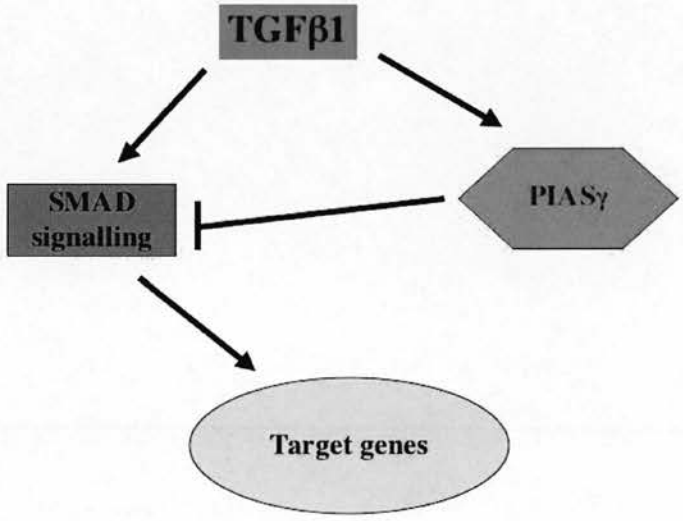


Figure 6.1: Diagram depicting the hypothetical TGFβ1-mediated PIAS γ inhibition of SMAD transcriptional activity and therefore the potential target genes, PR and DKK. TGFβ1 upregulates PIAS γ, which inhibits SMAD transcriptional activity, and in turn this may account for the TGFβ1–mediated suppression of PR and DKK.

In Chapter 4, studies focused on the impact of TGFβ1 on gene expression of decidualisation marker proteins. The studies in this chapter were conducted using primary human ESCs, obtained from non-pregnant endometrium, treated with 8-Br-cAMP and MPA to induce a decidualised phenotype, and DSCs obtained from 1st

trimester decidua. As the studies in Chapter 3 had demonstrated that TGFβ1 was capable of inhibiting the mediators of decidualisation, the initial focus of the studies was to evaluate the effect of TGFβ1 on the classical decidualisation marker proteins; tissue factor (TF) (Lockwood, Krikun et al. 1994), IGFBP-1 (Irwin, Kirk et al. 1989) and dPRL (Maslar and Riddick 1979). We report that TGFβ1 significantly downregulated mRNA expression of all three decidualisation markers (Figures 4.1, 4.3, and 4.5) and markedly suppressed mature protein release of both IGFBP-1 (Figure 4.2) and dPRL (Figure 4.4). These findings are in agreement with a number of studies reporting a marked inhibitory effect of TGFβ1 on IGFBP-1 protein secretion and basal and stimulated PRL secretion (Delidow, Billis et al. 1991; Jikihara and Handwerger 1994; Kubota, Taguchi et al. 1997; Minami and Sarkar 1997; Coya, Alvarez et al. 1999; Mazella, Tang et al. 2004). However, it must be noted that in the studies presented herein, the expected mRNA/protein kinetics were not observed. This may be attributed to several possibilities; firstly, the small reduction in IGFBP-1 and dPRL mRNA may be sufficient to result in a large change in protein release, secondly, TGFβ1 may be acting at a transcript level to produce greater levels of mRNA, whilst altering the RNA stability to prevent a rapid downregualtion of the mRNA expression. Furthermore, TGFβ1 may be altering translation of the RNA code thereby impacting on the protein synthesis, or TGFβ1 may be acting directly to impede the post-translational folding of the protein, thereby impacting the export or transport of the protein into the supernatant.

It was previously suggested that the long-term effect of TGFβ1 on inhibition of PRL expression might be attributed to decreased cAMP levels (Tanaka, Miyazaki et al. 1993; Herrlich, Kuhn et al. 1996; Telgmann, Maronde et al. 1997) as the dPRL promoter has previously been shown to contain a critical region located between –332 bp and –270 bp relative to the start site that is activated by 8-Br-cAMP and PKA (Telgmann, Maronde et al. 1997; Telgmann and Gellersen 1998). The data presented herein has shown for the first time that TGFβ1 abrogates the transactivation potential of the dPRL promoter-reporter, by more than 50%, acting at the minimal PKA-sensitive decidual PRL promoter construct (dPRL-332/Luc), in response to cAMP-

treated endometrial stromal cells (Figure 4.16). This suggests that TGFβ1 acts upon the region critical for successful transcription.

In contrast to the findings of the present studies, Celikkanat *et al* (Celikkanat, Atac et al. 2000), have reported that TGFβ1 is without effect on the differentiation of human ESC and on the production of dPRL, however their data are based on studies where cells were not exposed to any decidualising stimuli. Interestingly, Kim *et al* (Kim, Park et al. 2005), propose that TGFβ1 potentiates the decidualisation process in ESC and report that decidualisation is a progesterone-independent event, which is not only in contrast to the studies presented in this thesis, but also with every other publication studying decidualisation which have characterised it as a progesterone-dependent process.

The cells in our study were obtained from non-pregnant endometrium and decidualised *in vitro* and are therefore a model for the cells that decidualise in the absence of a blastocyst during the normal cycle. Previous studies have sought to identify an *in vitro* model better placed to study the effects and induction or inhibition of decidualisation (Schatz and Lockwood 1993). For this purpose 1st trimester decidual cells have been cultured and are reported to retain key morphological features indicative of their decidualised *in vivo* state (Schatz, Papp et al. 1994). In the current study, experiments to determine if stromal cells from 1st trimester decidua de-differentiate demonstrated that although the cells are seen to de-differentiate morphologically (Figure 4.10) and release reduced concentrations of the classic decidualisation marker, IGFBP-1, there is still a notable production of IGFBP-1, even after prolonged culture in the absence of decidualising stimuli (Figure 4.9). Furthermore, DSCs from gestations > 10 weeks release significantly more IGFBP-1 and take longer to demonstrate reduced IGFBP-1 release (Figure 4.9, panel B). Reduced recovery of IGFBP-1 from culture media may reflect a number of changes during the culture period. For example, there may be a progenitor cell present, which can proliferate and overgrow DSC in culture or alternatively in the samples from > 10 week gestation, cells may be present that have returned to a proliferative activity, even though they have been decidualised *in vivo*, and have

become the confluent cell type in culture. Furthermore, the studies reported in this thesis demonstrate that DSCs re-decidualised faster than ESC when treated in parallel, and displayed an increased sensitivity to MPA (Figures 4.12 – 4.14), implying the presence of a potential "decidualised phenotype memory" when cells are decidualised *in vivo*. These findings would seem to be in agreement with those of Oliver *et al* who used DSCs to analyse SMAα expression (Oliver, Montes et al. 1999), as these authors reported that the cells required exposure to progesterone *in vitro* before the cells would present a decidualised phenotype (Oliver, Montes et al. 1999).

In addition, the present studies have demonstrated that TGFβ1 markedly inhibits the expression of the classical decidualisation marker protein mRNAs in DSCs. However, this downregulation was delayed by at least 24 h as compared to TGFβ1-treated ESC (Figures 4.15, 4.17, 4.19), and was not reflected by a reduction in mature protein secretion in DSC (Figures 4.16 and 4.19), implying that decidualisation *in vivo*, confers some resistance to the actions of TGFβ1. The different response in DSCs as compared to ESCs is in contrast to findings by Jikihara *et al*, who published that TGFβ1-treatment significantly suppressed dPRL mRNA and protein in term decidual fibroblasts (Jikihara and Handwerger 1994). This discrepancy in TGFβ1 response in cells obtained from non-pregnant endometrium and those which had been decidualised in the presence of a blastocyst and increasing concentrations of hCG in the 1st trimester of pregnancy may contribute to an increase in cellular protection against potential pathogenic cytokines and growth factors in pregnant endometrium. Or, the role of TGFβ1 in pregnant endometrium may differ from that in non-pregnant, pre-menstrual endometrium, which would explain data reporting high expression of TGFβ1 in 1st trimester decidua without any detrimental effect to pregnancy (Lysiak, Hunt et al. 1995; Jokhi, King et al. 1997; Bennett, Lagoo-Deenadayalan et al. 1999; Simpson, Robson et al. 2002).

These studies have demonstrated that TGFβ1 is an important growth factor capable of modulating mediators of decidualisation resulting in suppression of the

decidualisation marker proteins (Figure 6.3). The results obtained suggest that ESCs isolated from non-pregnant endometrium are a better model than DSCs isolated from 1st trimester decidua for evaluating the events which occur in the premenstrual endometrium and suggest that local TGFβ1-signalling coordinates de-differentiation of endometrial stromal compartment and tissue remodelling associated with menstruation. It is likely that mass activation of TGFβ1, in response to falling progesterone levels, would elicit a rapid response as seen in the present *in vitro* study, and possibly contribute to the cascade of events involved in menstruation. Indeed, a previous study (Johannisson, Oberholzer et al. 1989) postulated that progesterone withdrawal alone could not be responsible for the plethora of changes observed within the endometrium during the late secretory and pre-menstrual phases of the cycle. Therefore, the action of TGFβ1 opposing progesterone-induced responses, for example by the induction of DKK and reduced expression of decidualisation marker proteins (Figure 6.3), serves to provide a rationale for the efficacy of progesterone withdrawal.

TGFβ1 is also responsible for stimulating expression of TIMPs and augmenting synthesis of collagen and fibronectin, both matrix proteins (Huang, Wen et al. 1998). Therefore, TGFβ1 is proposed to act by limiting MMP activity and stabilising the tissue, preventing aberrant breakdown of the endometrium during the secretory phase of the menstrual cycle in accord with cyclical expression of TGFβ1 (Marshburn, Arici et al. 1994) (Casslen, Sandberg et al. 1998). Overexpression and or misregulation of TGFβ1 activation may be implicated in the aetiology of endometrial disorders associated with menstruation, for example, aberrant menstrual blood loss and further investigation into this possibility is warranted.

In Chapter 5, the studies focused on the phenotypically unique uNK cells that are seen to increase in secretory phase endometrium and 1st trimester decidua of pregnancy (King, Wellings et al. 1989; Loke and King 1997). At present the lineage, origin and mechanism underlying the postovulatory expansion of uNK cells remains to be established. The exact functions of these uNK cells in humans have yet to be elucidated, but may involve a role in implantation and placentation (Loke and King

1995; Loke and King 1997; King, Burrows et al. 1998; King 2000; Loke and King 2000; Loke and King 2000). A very recent study has reported that uNK cells, but not peripheral blood-derived NK subsets, regulate trophoblast invasion both *in vivo* and *in vitro* by production of IL-8 and IP-10 chemokines (Hanna, Goldman-Wohl et al. 2006). Furthermore, the authors also reported that uNK cells isolated from 1st trimester decidua induced vascular growth in the decidua and interacted with the decidual stroma (Hanna, Goldman-Wohl et al. 2006). The variation in uNK cell number across the menstrual cycle suggests ovarian steroid regulation (King, Burrows et al. 1998; Dunn, Kelly et al. 2003; Dosiou and Giudice 2005). uNK cell expansion following the surge in pituitary-derived luteinising hormone (LH) across the progesterone-dominated secretory phase, and the association of uNK cell demise with falling levels of progesterone implicates progesterone as a central regulator of their growth and this is supported by evidence that ovariectomised women lack uNK cells (Loke and King 1995; Flynn, Byrne et al. 2000). However, search for evidence for a direct hormonal effect on uNK cells, failed to identify either PR or ERα in human uNK cells (King, Gardner et al. 1996; Stewart, Bulmer et al. 1998; Henderson, Saunders et al. 2003). The focal expression of many inflammatory mediators indicates that a more subtle mechanism, in addition to progesterone may be involved in uNK cell expression. At the beginning of the present studies no data had been compiled on the effect of LH or hCG on uNK cells. The data presented in this thesis demonstrated that low does of hCG inhibited IFNγ protein release by uNK cells isolated from 1st trimester decidua (Figure 5.7 and 6.2), which may provide a rationale for the regulation of trophoblast invasion, as limiting IFNγ production, in the very early stages of pregnancy may serve to explain the inability of uNK cells to kill semiallogenic foetal cells (Trundley and Moffett 2004). This may also be influenced by TGFβ1, which has recently been shown to suppress PBNK cell IFNγ (Laouar, Sutterwala et al. 2005; Meadows, Eriksson et al. 2006) and limit NK cell activity (Meadows, Eriksson et al. 2006). Furthermore, uPA and PAI-1, which regulate the activation of TGFβ1, have been detected in the subpopulation of extravillous cytotrophoblasts (Fisher and Damsky 1993). PAI-1 increases steadily during pregnancy (Houlihan, Knuppel et al. 1996), whereas the production of uPA by human trophoblasts is downregulated during the second trimester, paralleling the

decline in the invasiveness of trophoblast cells with gestational age (Chou, Zhu et al. 2002), implying that levels of activated TGFβ1 will be highest during the 1st trimester of pregnancy. This would be in agreement with several groups reporting high levels of TGFβ1 in 1st trimester decidua (Lysiak, Hunt et al. 1995; Jokhi, King et al. 1997; Bennett, Lagoo-Deenadayalan et al. 1999; Simpson, Robson et al. 2002), and may indicate that during the 1st trimester TGFβ1 may be acting to interact with uNK cells to facilitate and regulate trophoblast invasion. Additionally, Kovalevskaya *et al* (Kovalevskaya, Birken et al. 2002) reported that a hyperglycosylated form of hCG arises very early in pregnancy and is rapidly replaced by less glycosylated isoforms, showing that hCG glycoforms dynamically change as pregnancy progresses. This finding, in concert with the regulation of IFNγ, may provide a mechanism by which the embryo may transmit the information about its presence to maternal immune systems (Kosaka, Fujiwara et al. 2002).

hCG is considered to propagate its hormonal signal to target cells through the LH/hCG receptor, which has been reported to be expressed on ovarian granulosa cells, theca interna cells, luteal cells (Acevedo 2002) and macrophages from term placenta and late secretory phase non-pregnant endometrium (Zhang, Rao Ch et al. 2003), however in these studies no expression of the LH/hCG receptor was detected on the uNK cells (Figure 5.4). As the uNK cells in the current study were purified prior to culture (Figure 5.1, 5.2 and 5.3), any hCG-mediated responses would be a result of direct interaction with the uNK cell alone. We have demonstrated that hCG binding occurs even though LH/hCG receptor is not present (Figures 5.10 and 5.12). This finding is in agreement with studies analysing PB cells which failed to detect LH/hCG receptor expression in either PBNK or PBMN cells (Sulke, Jones et al. 1985; Kosaka, Fujiwara et al. 2002; van den Heuvel, Peralta et al. 2005) but that PBMN cells could be stimulated by exogenous hCG (Kosaka, Fujiwara et al. 2002).

A recent study has proposed that cell surface lectins, which can recognise carbohydrate moieties in glycosylated proteins, may play a role in the hCG-signaling pathway (Kosaka, Fujiwara et al. 2002). Recently, pituitary hormones such as LH and TSH were reported to interact with the macrophage mannose receptor (MMR)

(Fiete, Beranek et al. 1998; Simpson, Hitchen et al. 1999) and it was proposed that immune cells are involved in the clearance of these hormones (Simpson, Hitchen et al. 1999). Indeed recently, Laskarin *et al* have reported the presence of a functional MMR on early decidual macrophages (Laskarin, Cupurdija et al. 2005) implicating the receptor in maintaining homeostasis at the maternal-foetal interface (Laskarin, Cupurdija et al. 2005). We report, for the first time that the MMR is present on uNK cells, isolated from both non-pregnant endometrium and 1st trimester decidua (Figure 5.8 and 5.9). Further to this, we have found that cells treated with hCG show colocalisation of hCG with the MMR (Figure 5.12). This binding may be facilitated by terminal sialylation of the carbohydrates side chains on the MMR, which allows oligomerisation of the MMR, resulting in multiple CR domains, providing a possible binding site for the glycosylated hormones, such as LH and hCG (Su, Bakker et al. 2005).

The studies herein also provide non-quantitative evidence (photographic analysis, Figure 5.15) that uNK cells proliferate in response to hCG-treatment. This is in contrast with studies showing that culture with hCG did not induce $CD56^{Bright}$ cell proliferation, in an *in vitro* culture system where the NK cells were co-cultured with endometrial stromal cells (Inoue, Kanzaki et al. 1996). This putative LH/hCG-mediated mechanism for regulation of uNK cell expansion and/or function in late-secretory phase endometrium and 1st trimester pregnancy may be in concert with the actions of progesterone acting on the ESC, which retains its PR throughout the cycle (Wang, Critchley et al. 1998) (Figure 6.2). It is likely therefore that no one molecule is responsible for the unique expression and actions of the uNK cells, but that physiological endometrial changes mediated by paracrine hormonal-cellular signalling may orchestrate the dynamic and complex milieu of the uterine environment in which an immune-endocrine network involving hCG and uNK cells exists.

6.3 Future studies

The studies presented in this thesis have shown that, in the culture system used, TGFβ1 inhibits PR, DKK and the expression of decidualisation marker proteins;

tissue factor, IGFBP-1 and prolactin. Although we showed that TGFβ1 is capable of inducing SMAD phosphorylation, it remains to be identified whether downregulation of the aforementioned genes is SMAD-dependent. Knockdown of SMAD 4 expression in primary ESCs was attempted but the non-targeting siRNA sequence evoked a reduction in the mRNA expression of both PR and PRL, preventing any conclusive evidence to determine the signalling pathway of TGFβ1-mediated suppression of PR and PRL. Further experiments utilising different non-targeting siRNA sequences are required and should yield interesting results to conclusively answer this question.

In the culture system using DSCs isolated from 1st trimester, whether reduced expression of decidualising markers is due to de-differentiation or over proliferation of a progenitor cell needs to be elucidated. A further possibility is that some of the cells were not fully decidualised *in vivo,* prior to isolation, and may have proliferated *in vitro,* overgrowing the fully decidualised non-proliferative cells. To answer these possibilities, FACS analysis should be conducted to determine if a subset of cells are present which may act as progenitor cells, e.g. CD34 positive. Additionally, IHC may be performed; immunolocalising tissue factor antigens present on the cells to determine if some remain non-decidualised.

uNK cells appeared to proliferate in response to hCG treatment, however, further studies to quantify any increase in cell number should be performed. Studies using Trypan blue™ exclusion staining and cell counts are necessary. Additionally, incubation of cells with Bromodeoxyuridine (BrdU), which is an analogue of thymidine and can be incorporated into the newly synthesised DNA of replicating cells (S-phase cells), could be used to detect whether the cells were actively replicating their DNA. Another potential effect of hCG treatment that was not analysed was hCG-induced migration of uNK cells. Further work is required to design a migration assay, either using a commercially available assay or using a slide coated in an agarose-based medium to assess migration in response to hCG treatment.

Although colocalisation of hCG and the MMR on uNK cells was observed, functionality of the MMR was not ascertained. Treatment with mannose, which will compete for binding sites on MMRs, will elucidate if blocking potential hCG-binding via the MMR prevents hCG binding to uNK cells. Furthermore, apart from one sample, all uNK cell preparations were isolated form 1st trimester decidua and had been exposed to hCG *in vivo*. Experiments using uNK cells isolated from non-pregnant late secretory phase endometrium would be necessary to deduce any physiological response from hCG treatment, as cells which have been exposed to hCG *in vivo* may have a limited response to the *in vitro* hCG treatment in the studies presented herein.

Another area for investigation is the interaction between the ESC and the uNK cell. Co-culture of the two cell types would be an ideal model for examining the effects of cell to cell interactions. Additional treatment with progesterone and hCG, either alone or in combination, would further advance the knowledge on uNK cell expansion in late-secretory phase endometrium and decidua of early pregnancy. This hypothesis is summarised in Figure 6.2.

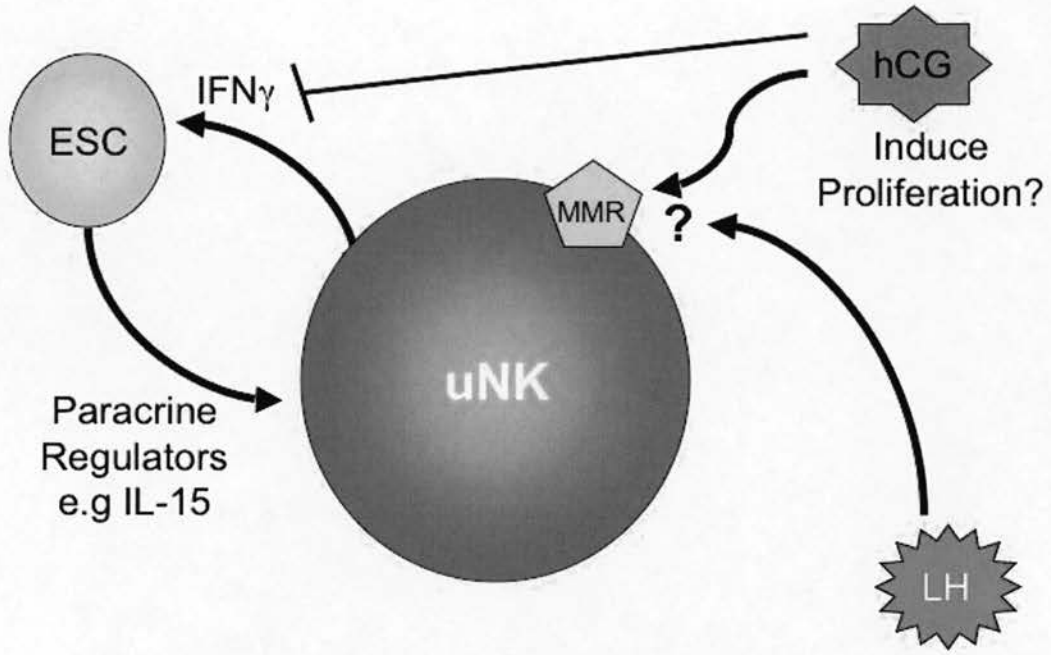


Figure 6.2: Diagram depicting the potential hCG-regulation of uNK expansion and function. uNK cells express the MMR, which may bind LH and or hCG to facilitate trophoblast invasion and cell expansion. Other functions may include interactions with other cell types such as the ESC, which may release paracrine regulators e.g. IL-15 in response to uNK derived IFNγ.

6.4 Conclusion

These studies have shown for the first time that TGFβ1 may potentiate progesterone withdrawal by acting as a progesterone receptor modulator, and may prevent the process of decidualisation by both stimulating the Wnt-signalling pathway via inhibition of DKK and suppressing the transactivation potential of the dPRL promoter (Figure 6.3). Additionally, the studies herein have demonstrated that a MMR receptor is present on uNK cells, and that this receptor may facilitate LH/hCG-mediated actions on uNK cells (Figure 6.2). Future studies will focus on firstly; the mechanism by which TGFβ1 mediates its actions to inhibit PR, DKK and the expression of decidualisation marker proteins, secondly; the reason for the suppression of IGFBP-1 protein release in DSCs isolated from 1st trimester decidua and thirdly; the role of MMR in the uNK cell and if LH and or hCG plays a

functional role in the expansion of uNK cells in the late secretory phase endometrium and decidua of early pregnancy. These findings may represent important factors that are critical in regulating both the progress of decidualisation and the withdrawal of progesterone prior to menstruation. In addition, the phenotypically unique uNK cell has been characterised further with regard to receptor expression and a possible regulator of uNK cell function elucidated.

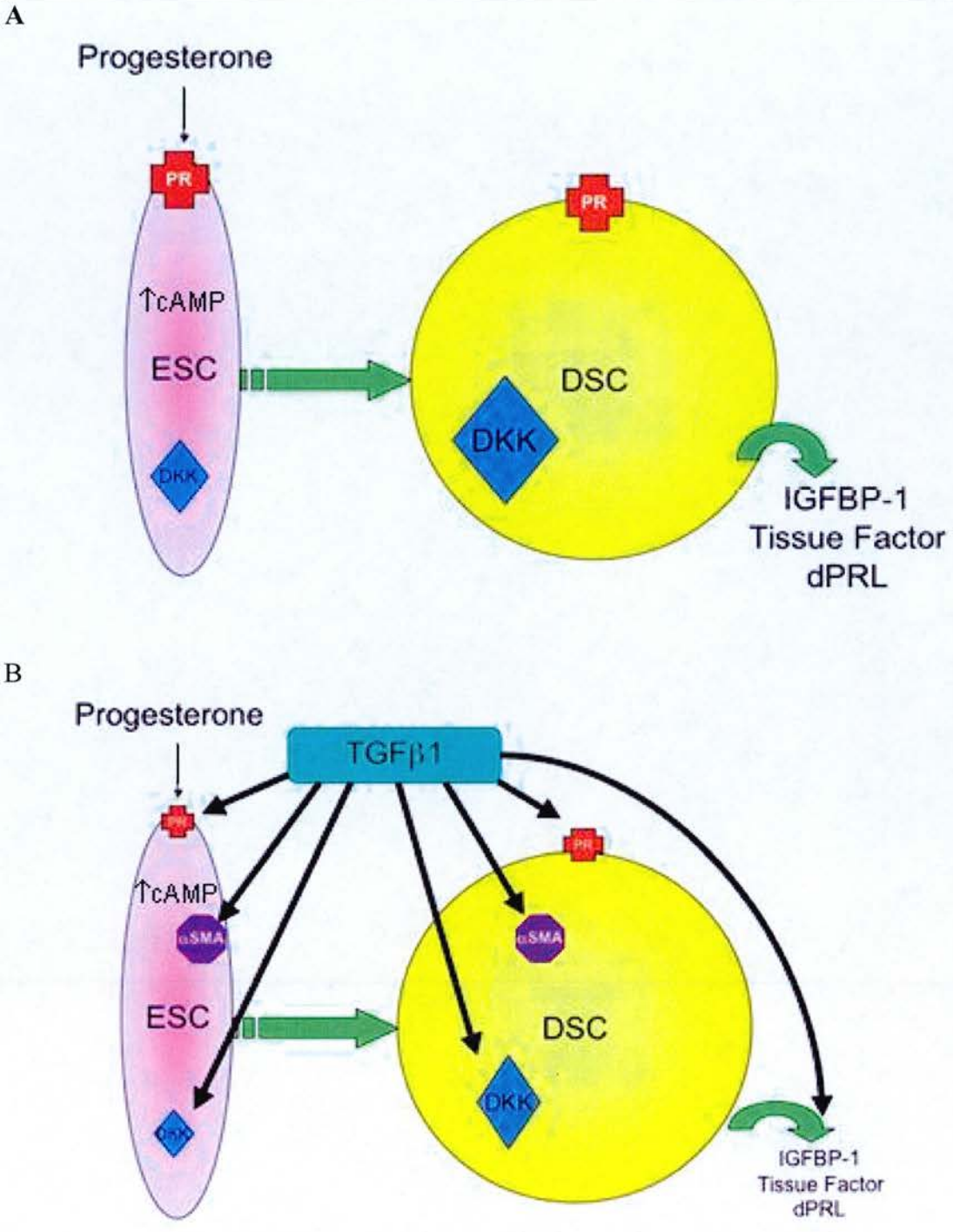


Figure 6.3: Diagram depicting the process of decidualisation *in vitro* and the response to treatment with TGFβ1. Panel A depicts an ESC decidualised *in vitro* with a "rounder" morphology and increased expression of DKK, the Wnt-signalling antagonist, and the classical decidualisation markers; IGFBP-1, tissue factor and dPRL. Panel B depicts the same cells treated with TGFβ1, which suppresses expression of PR and DKK in both non-decidualised and decidualised ESCs, increases αSMA expression and downregulates production of the decidualisation markers.

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8 Appendix

8.1 Patient information sheets and consent forms

LREC Number	Title of Study
05/S1104/12	Origins of Uterine NK cells
1994/6/17	Local Mediators in Menstruation
2003/6/40	Studies of natural antimicrobial agents from transformed uterine cells
1993/6/73	Study of the womb lining in both non-pregnant and pregnant women

Table 8.1: Patient information sheets and consent forms included in Appendix.

Study Number: LREC/05/S1104/12

Version 2: 4th April 2005

Patient information sheet – Group 1 (Hysterectomy)

Title of study – Origins of Uterine NK Cells

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please read the following information carefully and ask us if there is anything that is not clear, or if you would like any further information.

Purpose of the study

There are white blood cells in the lining of the womb called uterine natural killer cells (uNK). These cells are known to play an important role in key reproductive processes eg implantation of a fertilised egg in early pregnancy and menstruation (periods). However, the origins of these uNK cells is not known. It is possible that these uNK cells may originate from the womb lining itself or from the blood supply to the womb. To more fully investigate this we plan to study tissue samples from the lining of the womb (endometrium) from a group of women who are not pregnant (Groups 1 to 3) and those who are pregnant (Group 4). In addition, we also plan to look at white blood cells from women who are voluntarily donating blood at the Blood Transfusion Service (Group 5). This research will add to our knowledge and understanding of the function of the lining of the womb in early pregnancy and menstruation and may contribute to our understanding of the possible reasons for early pregnancy failure and menstrual problems.

What does the study involve ?

If you decide to take part in this study it would involve a short interview to enquire about your recent health and to ensure that the entry criteria to the study have been met. In addition, this study involves the collection of tissue samples from the lining of the womb and a single 5ml blood sample (a teaspoon) to measure hormone levels. Participation in this study is confined only to the day of your operation. The tissue samples are obtained with agreement from a gynaecological pathologist.

Tissue samples would be obtained from the lining of the womb after the womb has been removed. Therefore there would be no change to the planned surgical procedure and subsequent recovery from the operation. Some of the cells from the tissue sample of the lining of the womb may be cultured (grown), which is common practice in a laboratory, and may be stored for up to one year.

The information and samples will be stored and may be used in related studies in the future, addressing important and associated clinical research questions. At present these future studies cannot be specified in detail but may involve collaboration with laboratories working with Professor Hilary Critchley. The information and tissue samples will be given a unique code to protect your anonymity.

It is up to you if you decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time without giving a reason. A decision to withdraw at any time or a decision not to take part will not affect the standard of care you receive. This is non-therapeutic research from which you cannot expect to derive any benefit.

The research proposed has been reviewed by the Local Research Ethics Committee. If you are harmed from taking part in this study, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain about any aspect of the way that you have been approached or treated during the course of this study, the normal NHS complaints mechanisms may be available to you.

If you require any further information from a doctor who is not involved in this study you can contact Dr Christine West on 0131 242 2525.

Thank you for reading this information sheet.

Study Number: LREC/05/S1104/12

Version 2: 4th April 2005

Patient information sheet - Group 2 (Diagnostic Laparoscopy & Lap-sterilisation)

What does the study involve ?

If you decide to take part in this study it would involve a short interview to enquire about your recent health and to ensure that the entry criteria to the study have been met. In addition, this study involves the collection of tissue samples from the lining of the womb and a single 5ml blood sample (a teaspoon) to measure hormone levels. Participation in this study is confined only to the day of your operation.

A sample of the lining of the womb is obtained using a fine plastic sampler (pipelle). This is a routine procedure which involves passing the sampler through the neck of the womb (cervix) into the womb cavity. This procedure would be carried out during your planned surgery and whilst under general anaesthetic. We would not expect you to experience any untoward effects from the collection of this tissue sample and should not add to the discomfort that is normally expected following an operation.
In some cases, your doctor may have already explained that a tissue sample from the lining of the womb is required as part of your routine clinical care. If your gynaecologist decides that more tissue has been obtained than is required for clinical needs then the surplus tissue, with your permission, could be used for research purposes. Some of the cells from the tissue sample of the lining of the womb may be cultured (grown), which is common practice in a laboratory, and may be stored for up to one year.

Study Number: LREC/05/S1104/12

Version 2: 4th April 2005

Patient information sheet - Group 3 (Hysteroscopy)

What does the study involve ?

If you decide to take part in this study it would involve a short interview to enquire about your recent health and to ensure that the entry criteria to the study have been met. In addition, this study involves the collection of tissue samples from the lining of the womb and a single 5ml blood sample (a teaspoon) to measure hormone levels. Participation in this study is confined only to the day of your investigation.

Your gynaecologist has explained that a tissue sample from the lining of the womb is required as part of your routine clinical care which will be obtained using a fine plastic sampler (pipelle). This is a routine procedure which involves passing the sampler through the neck of the womb (cervix) into the womb cavity. If your

gynaecologist decides that more tissue has been obtained than is required for clinical needs then the surplus tissue, with your permission, could be used for research purposes. Some of the cells from the tissue sample of the lining of the womb may be cultured (grown), which is common practice in a laboratory, and may be stored for up to one year.

Study Number: LREC/05/S1104/12

Version 2: 4th April 2005

Patient information sheet - Group 4 (Termination of Pregnancy)

What does the study involve ?

If you decide to take part in this study it would involve a short interview to enquire about your recent health and to ensure that the entry criteria to the study have been met. In addition, this study involves the collection of tissue samples from the lining of the womb. Participation in this study is confined only to the day of your operation.

If you decide to take part in this study it would involve the collection of a sample of the pregnancy tissue which lines the womb. These tissue samples would have been removed as a matter of routine during the normal operation procedure. Therefore, your participation in this study would not change your planned operation or affect your recovery from the operation. Some of the cells from the tissue sample of the lining of the womb may be cultured (grown), which is common practice in a laboratory, and may be stored for up to one year.

Study Number: LREC/05/S1104/12

Patient Identification Number for this trial:

8.1.1 Version 3 18th October 2005

Department of Obstetrics and Gynaecology

CONSENT FORM

Title of Project: Origins of Uterine NK Cells

Name of Researcher:

Professor H O D Critchley
New Royal Infirmary of Edinburgh
Simpson Centre for Reproductive Health
51 Little France Crescent
Edinburgh EH16 4SA

Tel: 0131 242 2483
Or : 0131 242 6441

1. I confirm that I have read and understand the information sheet dated 4th April 2005(version 2) for Group of the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected.

3. I agree to the provision of clinically significant information to my General Practitioner.

4. I understand that my medical notes may be looked at by the researchers involved in the study or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

5. I agree that the sample I have given and the information gathered from me may be stored by Professor Hilary O D Critchley at the Centre for Reproductive Biology,

University of Edinburgh for possible use in future projects, as described in the attached information sheet. I understand that some of these projects may be carried out by researchers other than Professor Critchley's laboratory who ran the first project.

6. I understand that this is non-therapeutic research from which I cannot expect to derive any benefit.

7. I agree to take part in the above study.

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

LREC/1994/6/17

Version 3: Amended – 16th October 2002

Patient information sheet

Local mediators in menstruation

Lay title: Study of the local uterine factors involved in menstruation

In order for us to better understand the problems of menstruation it is essential that we have an opportunity to study womb-lining. Information gathered from this study will hopefully enable us to design new methods of managing this very common problem.

We would like your permission to collect a small sample (biopsy) from the lining of the womb during the time of your planned procedure for which you are currently attending the hospital.

The biopsy is collected with a slim plastic or metal instrument. The tissue collected remains anonymous. It will be processed in the laboratory in several different ways and will be used to investigate the function of the womb-lining. The tissue from the lining of the uterus (womb) will be broken down into cells and these cells will be used to examine the way in which menstruation (the monthly period) is controlled by hormones. This research will allow a better understanding of why periods are sometimes accompanied by distressing pain or a particularly large loss of blood. In order to carry out several experiments on the cells we will treat them so that they will grow in tissue culture. In addition we would like to collect a small sample of blood for the measurement of hormone levels.

I understand that the sample I have given for the above research study and the information gathered about me may be stored and consent to its inclusion in future research projects addressing the processes involved in regulation of the womb lining (endometrium). The information derived from such future studies will contribute to the understanding of the event of menstruation and therefore contribute to furthering knowledge when menstruation becomes problematic.

Involvement in this study will not involve any additional hospital attendance or an extension of your planned management. No additional discomfort or inconvenience will be experienced.

Your confidentiality will be strictly maintained throughout the study. If you volunteer then you are under no obligation or commitment and may withdraw at any time. Such a decision will not influence your treatment in any way.

Should you wish to contact an independent adviser concerning this study please feel free to contact: -

Dr C P West, Centre for Reproductive Health, Second Floor, Royal Infirmary of Edinburgh at Little France
51 Little France Crescent, Edinburgh EH16 4SA

For further information about the study, please contact: -
Professor H O D Critchley
New Royal Infirmary of Edinburgh
Simpson Centre for Reproductive Health
51 Little France Crescent
Edinburgh EH16 4SA

Tel: 0131 242 2670 or 0131 242 6441

Study Number: LREC/1994/6/17
Patient Identification Number for this trial:

CONSENT FORM

Department of Obstetrics and Gynaecology

Title of Project: Local mediators in menstruation
Study of the local uterine factors involved in menstruation

Name of Researcher:

Professor H O D Critchley
New Royal Infirmary of Edinburgh
Simpson Centre for Reproductive Health
51 Little France Crescent
Edinburgh EH16 4SA

Tel: 0131 242 2670
Or : 0131 242 6441

1. I confirm that I have read and understand the information sheet dated 16th October 2002 (version 3) for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time,without giving any reason, without my medical care or legal rights being affected.

3. I understand that sections of any of my medical notes may be looked at by responsible individuals or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

4. I agree that the sample I have given and the information gathered from me may be stored by Professor Hilary O D Critchley at the Centre for Reproductive Biology, University of Edinburgh for possible use in future projects, as described in the attached information sheet. I understand that some of these projects may be carried

out by researchers other than Professor Critchley's laboratory who ran the first project.

5. I agree to take part in the above study.

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Amended: 16th October 2002

LREC/2003/6/40

Version 1: 21st October 2003
Patient information sheet

Study Title - Studies of natural antimicrobial agents from transformed uterine cells

You are being invited to take part in a research study. Before you decide to take part it is important for you to understand why the research is being done and what it will involve. Please read the following information carefully and ask us if there is anything that is not clear, or if you would like more information.

The purpose of this study is to understand more about the role of specific cells that make up the lining of the womb (endometrium) that are associated with helping to protect the reproductive tract from infection. The reproductive tract can be prone to infection and sadly many of these infections can go undetected. Infections can cause distressing conditions such as infertility and, in pregnancy, premature births. Therefore, it is important to study the cells from the lining of the womb in women who are pregnant and those who are not. This research will be beneficial by allowing us to more fully understand how the reproductive tract attempts to protect itself from infections.

To do these experiments it is essential to grow (culture) and treat these cells in the laboratory in a particular way which prolongs the cells ability to exist for longer than required for most cellular experiments. However, these cells will not be kept indefinitely. Cells can lose their ability to be useful after repeated experiments as over time the results obtained may become unreliable. Therefore the cells will be destroyed before this happens.

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

If you decide to take part in this study it will involve obtaining a blood sample to measure hormone levels and a tissue sample from the lining of the womb. The tissue sample is obtained using a fine plastic sampler (pipelle). This is a procedure which is carried out routinely in outpatient gynaecology clinics and theatres. This sample will be obtained whilst you are asleep (anaesthetised) at the time of your planned surgery.

The information and tissue sample will be stored and may be used in related research studies in the future. At present, these future studies cannot be specified in detail but

may involve collaboration with laboratories working with Professor Critchley. It may be possible that some of these laboratories will be in the UK and / or overseas.

Your confidentiality will be strictly maintained throughout the study. The information and the tissue sample will be given a unique code to protect your anonymity. Permission will be sought from your consultant regarding your inclusion in the study and with your permission your GP will receive a letter informing him / her of your participation. This study has been approved by the Local Research Ethics Committee.

If you wish further information from a doctor who is not involved in this study in any way you can contact Dr Christine West, Simpson Centre for Reproductive Health, Royal infirmary of Edinburgh, 51 Little France Crescent, Edinburgh, EH16 4SA Tel 0131 242 2698

Thank you for reading this information sheet.

Study Number: LREC/2003/6/40

Patient Identification Number for this trial:

CONSENT FORM

Department of Obstetrics and Gynaecology

Study Title

Studies of natural antimicrobial agents from transformed uterine cells

Name of Researcher:

Professor H O D Critchley
New Royal Infirmary of Edinburgh
Simpson Centre for Reproductive Health
51 Little France Crescent
Edinburgh EH16 4SA

Tel: 0131 242 2670
Or : 0131 242 6441

1. I confirm that I have read and understand the information sheet dated 21st October 2003 for the above study and have had the opportunity to ask questions.

3. I understand that my participation is voluntary and that I am free to withdraw at any time,without giving any reason, and without my medical care or legal rights being affected.

4. I agree to a letter being sent to my General Practioner about my participation in this study.

4. I agree to the provision of any clinically significant information to my General Practioner.

5 I understand that my medical notes may be looked at by the researchers involved in the study or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

6. I agree that the sample I have given and the information gathered from me may be stored by Professor Hilary O D Critchley at the Centre for Reproductive Biology, University of Edinburgh for possible use in future projects, as described in the attached information sheet. I understand that some of these projects may be carried out by researchers other than Professor Critchley's laboratory who ran the first project.

7. I understand that this is non-therapeutic research from which I cannot expect to derive any benefit.

8. I agree to take part in the above study.

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

LREC/1993/6/73

Version 2: Amended – 16th October 2002

Patient information sheet

Lay title: Study of the womb lining in both non-pregnant and pregnant women

If you are being admitted to hospital for: Termination of Pregnancy

In order for us to better understand the problems of menstruation and repeated pregnancy failure it is essential that we have an opportunity to study womb-lining in healthy fertile women who do not report problems with menstruation and who have conceived easily.

We would like your permission to collect a small sample (biopsy) from the lining of the womb during the occasion of your termination. We are only interested in the mother's womb lining; no fetal tissue will be collected.

The biopsy is collected with a slim plastic or metal instrument. The tissue collected remains strictly anonymous. It will be processed in the laboratory in several different ways and will be used to access a variety of aspects which influence the function of the womb-lining. In addition we would like to collect a small sample of blood, while you are asleep; for the measurement of hormone levels.

Involvement in this study will not involve any additional hospital attendance or an extension of your planned management. No additional discomfort or inconvenience will be experienced.

I understand that the sample I have given for the above research study and the information gathered about me may be stored and consent to its inclusion in future research projects addressing the processes involved in regulation of the womb lining (endometrium). The information derived from such future studies will contribute to the understanding of the event of menstruation and therefore contribute to furthering knowledge when menstruation becomes problematic.

Your confidentiality will be strictly maintained throughout the study. If you volunteer then you are under no obligation or commitment and may withdraw at any time. Such a decision will not influence your treatment in any way.

Should you wish to contact an independent adviser concerning this study please feel free to contact: -

Dr C P West, Centre for Reproductive Health, Second Floor, Royal Infirmary of Edinburgh at Little France
51 Little France Crescent, Edinburgh EH16 4SA

For further information about the study, please contact: -
Professor H O D Critchley
New Royal Infirmary of Edinburgh
Simpson Centre for Reproductive Health
51 Little France Crescent
Edinburgh EH16 4SA

Tel: 0131 242 2670 or 0131 242 6441

LREC/1993/6/73

Version 3: Amended – 16th October 2002

Patient information sheet

Lay title: Study of the womb lining in both non-pregnant and pregnant women

If you are attending hospital for: - Planned Sterilisation Procedure

We would like your permission to collect a small sample (biopsy) from the lining of the womb during the time of your planned sterilisation procedure for which you are currently attending the hospital.

The biopsy is collected with a slim plastic or metal instrument. The tissue collected remains strictly anonymous. It will be processed in the laboratory in several different ways and will be used to investigate the function of the womb-lining. The tissue from the lining of the uterus (womb) will be broken down into cells and these cells will be used to examine the way in which menstruation (the monthly period) is controlled by hormones. This research will allow a better understanding of why periods are sometimes accompanied by distressing pain or a particularly large loss of blood. In order to carry out several experiments on the cells we will treat them so that they will grow in tissue culture. In addition we would like to collect a small sample of blood for the measurement of hormone levels.

Study Number: LREC/1993/6/73

Patient Identification Number for this trial:

CONSENT FORM

Department of Obstetrics

and Gynaecology

Title of Project: Study of the womb lining in both non-pregnant and pregnant women

Name of Researcher:

Professor H O D Critchley
New Royal Infirmary of Edinburgh
Simpson Centre for Reproductive Health
51 Little France Crescent
Edinburgh EH16 4SA

Tel: 0131 242 2670
Or : 0131 242 6441

1. I confirm that I have read and understand the information sheet dated 16th October 2002 for the above study and have had the opportunity to ask questions.

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I understand that sections of any of my medical notes may be looked at by responsible individuals or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

4. I agree that the sample I have given and the information gathered from me may be stored

by Professor Hilary O D Critchley at the Centre for Reproductive Biology, University of Edinburgh for possible use in future projects, as described in the attached information sheet. I understand that some of these projects may be carried out by researchers other than Professor Critchley's laboratory who ran the first project.

5. I agree to take part in the above study.

Name of Patient	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes
Amended: 16th October 2002